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(54) NOVEL DNA POLYMERASE

(57) The present invention relates to a DNA polymerase possesses the properties of 1) exhibiting higher polymerase activity when assayed by using as a substrate a complex resulting from primer annealing to a single stranded template DNA, as compared to the case where an activated DNA is used as a substrate; 2) possessing a 3'→5' exonuclease activity; 3) being capable of amplifying a DNA fragment of about 20 kbp, in the case where polymerase chain reaction (PCR) is carried out using λ-DNA as a template. It also relates to a DNA polymerase-constituting protein; a DNA containing the base sequence encoding thereof; and a method for producing the DNA polymerase. The present invention provides a novel DNA polymerase possessing both a high primer extensibility and a 3'→5' exonuclease activity.

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Description**TECHNICAL FIELD**

5 The present invention relates a DNA polymerase which is useful for a reagent for genetic engineering, a method for producing the same, and a gene encoding thereof.

BACKGROUND ART

10 DNA polymerases are useful enzymes for reagents for genetic engineering, and the DNA polymerases are widely used for a method for determining a base sequence of DNA, labeling, a method of site-directed mutagenesis, and the like. Also, thermostable DNA polymerases have recently been remarked with the development of the polymerase chain reaction (PCR) method, and various DNA polymerases suitable for the PCR method have been developed and commercialized.

15 Presently known DNA polymerases can be roughly classified into four families according to amino acid sequence homologies, among which family A (pol I type enzymes) and family B (α type enzymes) account for the great majority. Although DNA polymerases belonging to each family generally possess mutually similar biochemical properties, detailed comparison reveals that individual DNA polymerase enzymes differ from each other in terms of substrate specificity, substrate analog incorporation, degree and rate for primer extension, mode of DNA synthesis, association of
20 exonuclease activity, optimum reaction conditions of temperature, pH and the like, and sensitivity to inhibitors. Thus, those possessing especially suitable properties for the respective experimental procedures have been selectively used of all available DNA polymerases.

DISCLOSURE OF INVENTION

25 An object of the present invention is to provide a novel DNA polymerase not belonging to any of the above families, and possessing biochemical properties not owned by any of the existing DNA polymerases. For example, primer extension activity and 3'→5' exonuclease activity are considered as mutually opposite properties, and none of the existing DNA polymerase enzymes with strong primer extension activity possess 3'→5' exonuclease activity, which is an important proofreading function for DNA synthesis accuracy. Also, the existing DNA polymerases possessing excellent proof-
30 reading functions are poor in primer extension activity. Therefore, development of a DNA polymerase possessing both potent primer extension activity and potent 3'→5' exonuclease activity would significantly contribute to DNA synthesis *in vitro*.

35 Another object of the present invention is to provide a method for producing the novel DNA polymerase mentioned above.

A still another object of the present invention is to provide a gene encoding the DNA polymerase of the present invention.

40 As a result of extensive investigation, the present inventors have found genes of the novel DNA polymerase from hyperthermophilic archaeobacterium *Pyrococcus furiosus*, followed by cloning of the above genes, and have clarified that two kinds of novel proteins possess a novel DNA polymerase activity exhibiting the activity under coexistence of the above two kinds of proteins. Furthermore, the present inventors have prepared a transformant into which the above genes are introduced, and have succeeded in mass-producing the complex type DNA polymerase.

Accordingly, the gist of the present invention is as follows:

45 [1] A DNA polymerase characterized in that the DNA polymerase possesses the following properties:

1) exhibiting higher polymerase activity when assayed by using as a substrate a complex resulting from primer annealing to a single stranded template DNA, as compared to the case where an activated DNA is used as a substrate;

50 2) possessing a 3'→5' exonuclease activity;

3) being capable of amplifying a DNA fragment of about 20 kbp, in the case where polymerase chain reaction (PCR) is carried out using λ -DNA as a template under the following conditions:

PCR conditions:

55 (a) a composition of reaction mixture: containing 10 mM Tris-HCl (pH 9.2), 3.5 mM MgCl₂, 75 mM KCl, 400 μ M each of dATP, dCTP, dGTP and dTTP, 0.01% bovine serum albumin, 0.1% Triton X-100, 5.0 ng/50 μ l λ -DNA, 10 pmole/50 μ l primer λ 1 (SEQ ID NO:8 in Sequence Listing), primer λ 11 (SEQ ID NO:9 in Sequence Listing),

and 3.7 units/50 μ l DNA polymerase;

(b) reaction conditions: carrying out a 30-cycle PCR, wherein one cycle is defined as at 98°C for 10 seconds and at 68°C for 10 minutes;

- 5 [2] The DNA polymerase according to the above item [1], characterized in that the DNA polymerase exhibits a lower error rate in DNA synthesis as compared to Taq DNA polymerase;
- [3] The DNA polymerase according to the above item [1] or [2], wherein the molecular weight as determined by gel filtration method is about 220 kDa or about 385 kDa;
- 10 [4] The DNA polymerase according to any one of the above items [1] to [3], characterized in that the DNA polymerase exhibits an activity under coexistence of two kinds of DNA polymerase-constituting protein, a first DNA polymerase-constituting protein and a second DNA polymerase-constituting protein;
- [5] The DNA polymerase according to the above item [4], characterized in that the molecular weights of the first DNA polymerase-constituting protein and the second DNA polymerase-constituting protein are about 90,000 Da and about 140,000 Da as determined by SDS-PAGE, respectively;
- 15 [6] The DNA polymerase according to the above item [4] or [5], characterized in that the first DNA polymerase-constituting protein which constitutes the DNA polymerase according to the above item [4] or [5] comprises the amino acid sequence as shown by SEQ ID NO:1 in Sequence Listing, or is a functional equivalent thereof possessing substantially the same activity which results from deletion, insertion, addition or substitution of one or more amino acids in the amino acid sequence;
- 20 [7] The DNA polymerase according to the above item [4] or [5], characterized in that the second DNA polymerase-constituting protein which constitutes the DNA polymerase according to the above item [4] or [5] comprises the amino acid sequence as shown by SEQ ID NO:3 in sequence Listing, or is a functional equivalent thereof possessing substantially the same activity which results from deletion, insertion, addition or substitution of one or more amino acids in the amino acid sequence;
- 25 [8] The DNA polymerase according to item [4] or [5], characterized in that the first DNA polymerase-constituting protein which constitutes the DNA polymerase according to the above item [4] or [5] comprises the amino acid sequence as shown by SEQ ID NO:1 in Sequence Listing, or is a functional equivalent thereof possessing substantially the same activity which results from deletion, insertion, addition or substitution of one or more amino acids in the amino acid sequence, and that the second DNA polymerase-constituting protein which constitutes the DNA
- 30 polymerase according to the above item [4] or [5] comprises the amino acid sequence as shown by SEQ ID NO:3 in Sequence Listing, or is a functional equivalent thereof possessing substantially the same activity which results from deletion, insertion, addition or substitution of one or more amino acids in the amino acid sequence;
- [9] A first DNA polymerase-constituting protein which constitutes the DNA polymerase according to the above item [4] or [5], wherein the first DNA polymerase-constituting protein comprises the amino acid sequence as shown by
- 35 SEQ ID NO:1, or an amino acid sequence resulting from deletion, insertion, addition or substitution of one or more amino acids in the amino acid sequence, as a functional equivalent thereof possessing substantially the same activity;
- [10] A second DNA polymerase-constituting protein which constitutes the DNA polymerase according to the above [4] or [5], wherein the second DNA polymerase-constituting protein comprises the amino acid sequence as shown
- 40 by SEQ ID NO:3, or an amino acid sequence resulting from deletion, insertion, addition or substitution of one or more amino acids in the amino acid sequence as a functional equivalent thereof possessing substantially the same activity;
- [11] A DNA containing a base sequence encoding the first DNA polymerase-constituting protein according to the above item [9], characterized in that the DNA comprises an entire sequence of a base sequence encoding the
- 45 amino acid sequence as shown by SEQ ID NO:1 in Sequence Listing, or a partial sequence thereof, or that the DNA encodes a protein having an amino acid sequence resulting from deletion, insertion, addition or substitution of one or more amino acids in the amino acid sequence of SEQ ID NO:1 and possessing a function as the first DNA polymerase-constituting protein;
- [12] A DNA containing a base sequence encoding the first DNA polymerase-constituting protein according to the above items [9], characterized in that the DNA comprises an entire sequence of the base sequence as shown by
- 50 SEQ ID NO:2 in Sequence Listing or a partial sequence thereof, or that the DNA comprises a base sequence capable of hybridizing thereto under stringent conditions;
- [13] A DNA containing a base sequence encoding the second DNA polymerase-constituting protein according to the above item [10], characterized in that the DNA comprises an entire sequence of a base sequence encoding the
- 55 amino acid sequence as shown by SEQ ID NO:3, or a partial sequence thereof, or that the DNA encodes a protein having an amino acid sequence resulting from deletion, insertion, addition or substitution of one or more amino acids in the amino acid sequence of SEQ ID NO:3 and possessing a function as the second DNA polymerase-constituting protein;

[14] A DNA containing a base sequence encoding the second DNA polymerase-constituting protein according to the item [10], characterized in that the DNA comprises an entire sequence of the base sequence as shown by SEQ ID NO:4 in Sequence Listing or a partial sequence thereof, or that the DNA comprises a base sequence capable of hybridizing thereto under stringent conditions;

[15] A method for producing a DNA polymerase, characterized in that the method comprises culturing a transformant containing both gene encoding the first DNA polymerase-constituting protein according to the above item [11] or [12], and gene encoding the second DNA polymerase-constituting protein according to the above item [13] or [14], and collecting the DNA polymerase from the resulting culture; and

[16] A method for producing a DNA polymerase, characterized in that the method comprises culturing a transformant containing gene encoding the first DNA polymerase-constituting protein according to the above item [11] or [12], and a transformant containing gene encoding the second DNA polymerase-constituting protein according to the above item [13] or [14], separately; mixing DNA polymerase-constituting proteins contained in the resulting culture; and collecting the DNA polymerase.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows a restriction endonuclease map of the DNA fragment inserted into the cosmid Clone No. 264 and the cosmid Clone No. 491 obtained in Example 1.

Figure 2 shows a restriction endonuclease map of an *Xba*I-*Xba*I DNA fragment inserted into a plasmid pFU1001.

Figure 3 is a graph for an optimum pH of the DNA polymerase of the present invention.

Figure 4 is a graph for a heat stability of the DNA polymerase of the present invention.

Figure 5 is a graph for a 3'→5' exonuclease activity of the DNA polymerase of the present invention.

Figure 6 is an autoradiogram for a primer extension activity of the DNA polymerase of the present invention.

BEST MODE FOR CARRYING OUT THE INVENTION

(1) DNA Polymerase of Present Invention and Constituting Proteins Thereof

An example of the DNA polymerase of the present invention has the following properties:

- 1) exhibiting higher polymerase activity when assayed by using as a substrate a complex resulting from primer annealing to a single stranded template DNA, as compared to the case where an activated DNA (DNase I-treated calf thymus DNA) is used as a substrate;
- 2) possessing a 3'→5' exonuclease activity;
- 3) optimum pH being between 6.5 and 7.0 (in potassium phosphate buffer, at 75°C);
- 4) exhibiting a remaining activity of about 80% after heat treatment at 80°C for 30 minutes;
- 5) being capable of amplifying a DNA fragment of about 20 kbp, in the case where polymerase chain reaction (PCR) is carried out using λ -DNA as a template under the following conditions:

PCR conditions:

- (a) composition of reaction mixture: containing 10 mM Tris-HCl (pH 9.2), 3.5 mM MgCl₂, 75 mM KCl, 400 μ M each of dATP, dCTP, dGTP and dTTP, 0.01% bovine serum albumin (BSA), 0.1% Triton X-100, 5.0 ng/50 μ l λ -DNA, 10 pmole/50 μ l primer λ 1 (SEQ ID NO:8 in Sequence Listing), primer λ 11 (SEQ ID NO:9 in Sequence Listing), and 3.7 units/50 μ l DNA polymerase. Here, one unit of the DNA polymerase is defined as follows: Fifty microliters of a reaction mixture [20 mM Tris-HCl (pH 7.7), 15 mM MgCl₂, 2 mM 2-mercaptoethanol, 0.2 mg/ml activated DNA, 40 μ M each of dATP, dCTP, dGTP and dTTP, 60 nM [³H]-dTTP (manufactured by Amersham)], containing a sample to assay activity, is reacted at 75°C for 15 minutes. A 40 μ l portion of this reaction mixture is spotted onto a DE paper (manufactured by Whatman) and washed with 5% Na₂HPO₄ five times. Thereafter, the remaining radioactivity on the DE paper is measured using a liquid scintillation counter, and the amount of the enzyme causing the incorporation of 10 nmol of [³H]-dTTP per 30 minutes into a substrate DNA is defined as one unit of the enzyme; and
- (b) PCR conditions: carrying out a 30-cycle PCR, wherein one cycle is defined as at 98°C for 10 seconds and at 68°C for 10 minutes; and

- 6) The DNA polymerase of the present invention is superior to the Taq DNA polymerase in terms of both primer extension activity and accuracy of DNA synthesis. Specifically, the DNA polymerase of the present invention is superior to the Taq DNA polymerase, a typical thermostable DNA polymerase (e.g., TaKaRa Taq, manufactured by Takara Shuzo Co., Ltd.), in terms of primer extension properties in DNA synthesis reaction, for instance, DNA

strand length capable of DNA amplification by PCR method, and accuracy of DNA synthesis reaction (low error rate in DNA synthesis).

The DNA polymerase of the present invention is an enzyme constituted by two kinds of proteins, wherein a molecular weight of the DNA polymerase of the present invention is about 220 kDa or about 385 kDa, as determined by gel filtration, and also shown by two bands corresponding to about 90,000 Da and about 140,000 Da on SDS-PAGE, respectively. The protein of about 90,000 Da (corresponding to ORF3 as described below) is herein referred to as the first DNA polymerase-constituting protein, and the protein of about 140,000 Da (corresponding to ORF4 as described below) is herein referred to as the second DNA polymerase-constituting protein. It is assumed that in the DNA polymerase of the present invention, the first DNA polymerase-constituting protein and the second DNA polymerase-constituting protein are non-covalently bonded to form a complex in a molar ratio of 1:1 or 1:2.

The first DNA polymerase-constituting protein which constitutes the DNA polymerase of the present invention may comprise the amino acid sequence shown by SEQ ID NO:1 in Sequence Listing, or may be a functional equivalent possessing substantially the same activity. Also, the second DNA polymerase-constituting protein may comprise the amino acid sequence shown by SEQ ID NO:3 in Sequence Listing, or may be a functional equivalent possessing substantially the same activity.

The term "a functional equivalent" as described in the present specification is defined as follows. A protein existing in nature can undergo mutation, such as deletion, insertion, addition and substitution, of amino acids in an amino acid sequence thereof owing to modification reaction and the like of the protein itself *in vivo* or during purification, besides causation such as polymorphism and mutation of the genes encoding it. However, it has been known that there are some proteins which exhibit substantially the same physiological activities or biological activities as a protein without mutation. Those proteins having structural differences as described above without recognizing any significant differences of the functions and the activities thereof, are referred to as "a functional equivalent." Here, the number of mutated amino acids is not particularly limited, as long as the resulting protein exhibits substantially the same physiological activities or biological activities as a protein without mutation. Examples thereof include one or more of mutations, for instance, one or several mutations, more specifically one to about ten mutations (such as deletion, insertion, addition and substitution) and the like.

The same can be said for the resulting proteins in the case where the above mutation is artificially introduced into the amino acid sequence of a protein. In this case, more diverse mutants can be prepared. For example, although the methionine residue at the N-terminus of a protein expressed in *Escherichia coli* is reportedly often removed by the action of methionine aminopeptidase, since the methionine residue is not removed perfectly depending on the kinds of proteins, those having methionine residue and those without methionine residue can be both produced. However, the presence or absence of the methionine residue does not affect protein activity in most cases. It is also known that a polypeptide resulting from substitution of a particular cysteine residue with serine in the amino acid sequence of human interleukin 2 (IL-2) retains IL-2 activity [Science, 224, 1431 (1984)].

In addition, during the production of a protein by genetic engineering, the desired protein is often expressed as a fusion protein. For example, purification of the desired protein is facilitated by adding the N-terminal peptide chain derived from another protein to the N-terminus of the desired protein to increase the amount of expression of the desired protein, or by adding an appropriate peptide chain to the N- or C-terminus of the desired protein, expressing the protein, and using a carrier having affinity for the peptide chain added. Accordingly, a DNA polymerase having an amino acid sequence which has a partial difference with that of the DNA polymerase of the present invention is within the scope of the present invention as "a functional equivalent," as long as it exhibits substantially the same activities as the DNA polymerase of the present invention.

(2) Gene of DNA Polymerase of Present Invention

The DNA encoding the first DNA polymerase-constituting protein which constitutes the DNA polymerase of the present invention includes a DNA comprising an entire sequence of the base sequence encoding the amino acid sequence as shown by SEQ ID NO:1 in Sequence Listing or a partial sequence thereof including, for instance, a DNA comprising an entire sequence of the base sequence as shown by SEQ ID NO:2 or a partial sequence thereof. Specifically, a DNA comprising a partial sequence of the base sequence encoding the amino acid sequence as shown by SEQ ID NO:1 including, for instance, the DNA comprising a partial sequence of the base sequence as shown by SEQ ID NO:2 in Sequence Listing, the base sequence encoding a protein possessing a function of the first DNA polymerase-constituting protein is also included in the scope of the present invention. Also, in the amino acid sequence as shown by SEQ ID NO:1, the above DNA also includes a DNA encoding a protein comprising an amino acid sequence resulting from deletion, insertion, addition, substitution and the like of one or several amino acids, the protein possessing a function of the first DNA polymerase-constituting protein. Furthermore, a base sequence capable of hybridizing to the above base sequences under the stringent conditions, the base sequence encoding a protein possessing a function of the first

DNA polymerase-constituting protein, is also included in the scope of the present invention. In addition, the DNA encoding the second DNA polymerase-constituting protein which constitutes the DNA polymerase of the present invention includes a DNA comprising an entire sequence of the base sequence encoding the amino acid sequence as shown by SEQ ID NO:3 in Sequence Listing or a partial sequence thereof including, for instance, a DNA comprising an entire sequence of the base sequence as shown by SEQ ID NO:4 in Sequence Listing or a partial sequence thereof. Specifically, the DNA comprising a partial sequence of the base sequence encoding the amino acid sequence as shown by SEQ ID NO:3, for instance, the DNA comprising a partial sequence of the base sequence as shown by SEQ ID NO:4 in Sequence Listing, the base sequence encoding a protein possessing a function of the second DNA polymerase-constituting protein, is also included in the scope of the present invention. Also, in the amino acid sequence as shown by SEQ ID NO:3, the above DNA also includes a DNA encoding a protein comprising an amino acid sequence resulting from deletion, insertion, addition, substitution and the like of one or several amino acids, the protein possessing a function of the second DNA polymerase-constituting protein. Furthermore, a base sequence capable of hybridizing to the above base sequences under the stringent conditions, the base sequence encoding a protein possessing a function of the second DNA polymerase-constituting protein, is also included in the scope of the present invention.

The term "protein possessing a function of the first DNA polymerase-constituting protein" or "protein possessing a function of the second DNA polymerase-constituting protein" herein refers to a protein possessing properties exhibiting a DNA polymerase activity with various physicochemical properties shown in the above items 1) to 6).

Here, the term "capable of hybridizing under the stringent conditions" refer to hybridizing to a probe, after incubating at 50°C for 12 to 20 hours in 6 × SSC (wherein 1 × SSC shows 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.5% SDS, 0.1% bovine serum albumin (BSA), 0.1% polyvinyl pyrrolidone, 0.1% Ficol 400, and 0.01% denatured salmon sperm DNA with the probe.

The term "DNA containing a base sequence encoding an amino acid sequence" described in the present specification will be explained. One to six kinds are known to exist for each amino acid with regards to a codon (triplet base combination) for designating a particular amino acid on the gene. Therefore, there can be a large number of DNA encoding an amino acid sequence, though depending on the amino acid sequence. In nature, genes do not always exist in stable forms, and it is not rare for genes to undergo mutations on a base sequence. There may be a case where mutations on the base sequence do not give rise to any changes in an amino acid sequence to be encoded (referred to as silent mutation). In this case, it can be said that different kinds of genes encoding the same amino acid sequence have been generated. The possibility, therefore, cannot be negated for producing a variety of genes encoding the same amino acid sequence after many generations of the organism even when a gene encoding a particular amino acid sequence is isolated.

Moreover, it is not difficult to artificially produce a variety of genes encoding the same amino acid sequence by means of various genetic engineering techniques. For example, when a codon used in the natural gene encoding the desired protein is used at a low frequency in the host in the production of the protein by genetic engineering, the amount of a protein expressed is sometimes low. In this case, high expression of the desired protein is achieved by artificially converting the codon into another one used at a high frequency in the host without changing the amino acid sequence encoded (for instance, Japanese Patent Laid-Open No. Hei 7-102146). As described above, it is, of course, possible to artificially produce a variety of genes encoding a particular amino acid sequence. Such artificially produced different polynucleotides are, therefore, included in the scope of the present invention, as long as the gene encodes the amino acid sequence disclosed in the present invention.

(3) Method for Producing DNA Polymerase of Present Invention

The present inventors have found genes of a novel DNA polymerase from a hyperthermophilic archaeobacterium, *Pyrococcus furiosus*, and cloned to clarify that the genes encode a novel DNA polymerase exhibiting its activity by the coexistence of two kinds of proteins on the genes. In the present invention, the DNA polymerase of the present invention can be mass-produced by preparing transformants incorporating the above genes. For this purpose, the transformant may be prepared by a process comprising culturing a transformant containing both the gene encoding the first DNA polymerase-constituting protein and the gene encoding the second DNA polymerase-constituting protein, and collecting the DNA polymerase from the resulting culture. Alternatively, the transformant may be prepared by a process comprising separately culturing a transformant containing the gene encoding the first DNA polymerase-constituting protein and a transformant containing the gene encoding the second DNA polymerase-constituting protein, mixing the DNA polymerase-constituting proteins contained in the resulting culture, and collecting the DNA polymerase therefrom.

Here, the phrase "transformant containing both the gene encoding the first DNA polymerase-constituting protein and the gene encoding the second DNA polymerase-constituting protein" may be a transformant resulting from co-transformation with two expression vectors containing the respective genes, or it may be a transformant prepared by recombining both genes into one expression vector to allow the respective proteins to be expressed.

(4) A cloning of the genes of the DNA polymerase of the present invention, an analysis of obtained clones, physico-chemical properties, activities, applicabilities to PCR method of expression product DNA polymerase, and the like are hereinafter described in detail.

5 The strain used for the present invention is not subject to particular limitation. Examples thereof include *Pyrococcus furiosus* DSM3638, as a strain belonging to the genus *Pyrococcus*. The above strain can be made available from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. In the case of culturing the above strain in an appropriate growth culture, preparing a crude extract from the resulting culture, and subjecting the crude extract to a polyacrylamide gel electrophoresis, since the present inventors found existences of several kinds of protein bands
10 showing DNA polymerase activity in the gel, it has been anticipated that the genes corresponding to these respective bands have existed. Specifically, the novel DNA polymerase gene and the product thereof can be cloned by the procedures exemplified below.

- 1) DNA is extracted from *Pyrococcus furiosus*;
- 15 2) The DNA obtained in 1) is digested with an appropriate restriction endonuclease, to prepare a DNA library with a plasmid, cosmid and the like, as a vector;
- 3) The library prepared in 2) is introduced into *Escherichia coli*, and a foreign gene is expressed to prepare a protein library in which crude extracts of the resulting clones are collected;
- 4) A DNA polymerase activity is assayed by using the protein library prepared in 3), and a foreign DNA is taken out
20 from the *Escherichia coli* clone which provides a crude extract having an activity;
- 5) The *Pyrococcus furiosus* DNA fragment contained in the plasmid or cosmid taken out is analyzed to narrow down the gene region encoding a protein exhibiting a DNA polymerase activity;
- 6) The base sequence of the region in which the protein exhibiting a DNA polymerase activity is presumably encoded is determined to deduce the primary structure of the protein; and
- 25 7) An expression plasmid is constructed to take a form which more easily allows the expression of the protein deduced in 6) in *Escherichia coli*, and the produced protein is purified and analyzed for the properties thereof.

The above DNA donor, *Pyrococcus furiosus* DSM3638, is a hyperthermophilic archaeobacterium, which is cultured at 95°C under anaerobic conditions. Known methods can be used as a method for disrupting grown cells followed by
30 extracting and purifying DNA, a method for digesting the obtained DNA with a restriction endonuclease and for other methods. Such methods are described in detail by in *Molecular Cloning: A Laboratory Manual*, 75-178, published by Cold Spring Harbor Laboratory in 1982, edited by T. Maniatis et al.

In the preparation of a DNA library, the triple helix cosmid vector (manufactured by Stratagene), for example, can be used. The DNA of *Pyrococcus furiosus* is partially digested with *Sau3AI* (manufactured by Takara Shuzo Co., Ltd.),
35 and the digested DNA is subjected to density gradient centrifugation to obtain the long DNA fragments. They are ligated to the *Bam*HI site of the above vector, followed by *in vitro* packaging. The respective transformants obtained from the DNA library thus prepared are separately cultured. After harvesting, cells are disrupted by ultrasonication, and the resulting disruption is heat-treated to inactivate the DNA polymerase from the host *Escherichia coli*. Thereafter, a supernatant containing a thermostable protein can be obtained by centrifugation. The above supernatant is named as
40 a cosmid protein library. By means of assaying the DNA polymerase activity using a portion of the supernatant, a clone that expresses the DNA polymerase derived from *Pyrococcus furiosus* can be obtained. DNA polymerase activity can be assayed using the known method described in *DNA Polymerase from Escherichia coli*, published by Harper and Row, edited by D.R. Davis, 263-276 (authored by C.C. Richardson).

One of the DNA polymerase genes of *Pyrococcus furiosus* has already been cloned and its structure clarified by
45 the present inventors, as described in *Nucleic Acids Research*, 21, 259-265 (1993). The translation product of the above gene is a polypeptide having a molecular weight of about 90,000 Da and consisting of 775 amino acids, and the amino acid sequence thereof clearly contains preserved sequences of the α -type DNA polymerases. In fact, since the DNA polymerase activity exhibited by this gene product is inhibited by aphidicolin, which is a specific inhibitor of α -type DNA polymerases, the above DNA polymerase is distinguishable from the DNA polymerase of the present invention.
50 Therefore, the above known gene out of the obtained clones exhibiting thermostable DNA polymerase activity can be removed by a process comprising digesting the cosmid contained in each clone, carrying out hybridization with the above gene as a probe, and selecting an unhybridizing clone. A restriction endonuclease map of the DNA insert can be prepared for the cosmid digested with the resulting clone containing the novel DNA polymerase gene. Next, a location of the DNA polymerase gene on the above DNA fragment can be determined by a process comprising dividing the
55 above DNA fragment into various regions on the basis of the obtained restriction endonuclease map, subcloning each region into a plasmid vector, introducing the resulting vector into *Escherichia coli*, and assaying the thermostable DNA polymerase activity exhibited therein. An *Xba*I-*Xba*I DNA fragment of about 10 kbp containing the DNA polymerase gene can be thus obtained.

The recombinant *Escherichia coli* harboring a plasmid incorporating the above DNA fragment exhibits a sufficient level of a DNA synthesis activity in the crude extract thereof even after treatment at 90°C for 20 minutes, while such an activity is not found in any plasmids without incorporating a DNA fragment. Therefore, it can be concluded that the information for producing a thermostable polymerase is present on the DNA fragment, and that a gene having the above information is expressed in the above *Escherichia coli*. The plasmid resulting from recombination of the DNA fragment into a pTV118N vector (manufactured by Takara Shuzo Co., Ltd.) is named as pFU1001. The *Escherichia coli* JM109 transformed with the above plasmid is named and identified as *Escherichia coli* JM109/pFU1001, has been deposited under accession number FERM BP-5579 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, of which the address is 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305, Japan, since August 11, 1995 (date of original deposit) under the Budapest Treaty.

The base sequence of the DNA fragment inserted in the plasmid pFU1001 can be determined by a conventional method, for instance, by the dideoxy method. Furthermore, regions capable of encoding a protein in the base sequence, i.e., open reading frames (ORFs), can be deduced by analyzing the resulting base sequence.

An 8,450 bp sequence in the base sequence of the *Xba*I-*Xba*I DNA fragment of about 10 kbp inserted in the plasmid pFU1001 is shown by SEQ ID NO:5 in Sequence Listing. In the base sequence, there are six consecutive ORFs, named as ORF1, ORF2, ORF3, ORF4, ORF5, and ORF6, respectively, naming from the 5' terminal side. FIG. 2 shows the restriction endonuclease map of the above *Xba*I-*Xba*I fragment and the location of the ORFs on the fragment (ORF1 to ORF6, from the left in the Figure).

A sequence showing homologies to any of known DNA polymerases was not found in any one of the above six ORFs. It should be noted, however, that on ORF1 and ORF2, there is a sequence homologous to the CDC6 protein found in *Saccharomyces cerevisiae*, or a sequence homologous to the CDC18 protein found in *Schizosaccharomyces pombe*. The CDC6 and the CDC18 are anticipated as proteins that are necessary for the cell cycle shift to the DNA synthesis phase (S phase) in yeasts, the proteins regulating initiation of the DNA replication. Also, the ORF6 has a sequence homologous to the RAD51 protein, known to act in DNA damage repair in yeasts and recombination in the somatic mitosis phase and in the meiosis phase in yeasts, and a sequence homologous to the Dmc1 protein, a meiosis phase-specific homolog to the RAD51 protein. The gene encoding the RAD51 protein is also known to be expressed at the cell cycle shift from the G1 to S phase. For the other ORFs, namely ORF3, ORF4, and ORF5, there have been no known proteins found to have a homologous sequence.

It is possible to determine from which of the above ORFs the thermostable DNA polymerase activity is derived by a process comprising preparing recombinant plasmids inserted with the respective DNA fragments deleting various regions, transforming a host with the plasmids, and assaying the thermostable polymerase activity of each transformant obtained. The transformant resulting from transformation with a recombinant plasmid inserted with a DNA fragment prepared by deleting ORF1 or ORF2, or deleting ORF5 or ORF6, from the above *Xba*I-*Xba*I DNA fragment of about 10 kbp retains the thermostable DNA polymerase activity, while those resulting from transformation with a recombinant plasmid inserted with a DNA fragment prepared by deleting ORF3 or ORF4 loses its activity. This fact predicts that the DNA polymerase activity is encoded by ORF3 or ORF4.

It is possible to determine by which of ORF3 and ORF4 the DNA polymerase is encoded by a process comprising preparing recombinant plasmids separately inserted with the respective ORFs, transforming a host with each recombinant plasmid, and assaying exhibition of a thermostable DNA polymerase activity in each transformant obtained. Unexpectedly, only very weak DNA polymerase activity is detected in a crude extract obtained from the transformant containing ORF3 or ORF4 alone. However, since a similar level of a thermostable DNA polymerase activity to that in the transformant containing both ORF3 and ORF4 can be obtained in the case where the two extracts are mixed, it is shown that the novel DNA polymerase of the present invention requires the actions of the translation products of the two ORFs. It is possible to find out whether the two proteins form a complex to exhibit the DNA polymerase activity, or one modifies the other to convert it to an active enzyme by determining the molecular weight of the DNA polymerase. The results of the determination of the molecular weight of the above DNA polymerase by gel filtration method demonstrate that the above two proteins form a complex.

The base sequence of ORF3 is shown by SEQ ID NO:2 in Sequence Listing, and the amino acid sequence of the ORF3-derived translation product, namely the first DNA polymerase-constituting protein as deduced from the base sequence, is shown by SEQ ID NO:1. The base sequence of ORF4 is shown by SEQ ID NO:4 in Sequence Listing, and the amino acid sequence of the ORF4-derived translation product, namely the second DNA polymerase-constituting protein as deduced from the base sequence, is shown by SEQ ID NO:3.

The DNA polymerase of the present invention can be expressed in cells by culturing a transformant resulting from transformation with a recombinant plasmid into which both ORF3 and ORF4 are introduced, for instance, *Escherichia coli* JM109/pFU1001, under usual culturing conditions, for instance, culturing in an LB medium (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl, pH 7.2) containing 100 µg/ml ampicillin. The above polymerase can be purified from the above cultured cells to the extent that only the two kinds of bands of nearly two kinds of the DNA polymerase-constituting pro-

teins are obtained in SDS-polyacrylamide gel electrophoresis (SDS-PAGE), by carrying out ultrasonication, heat treatment, and chromatography using an anionic exchange column (RESOURCE Q column, manufactured by Pharmacia), a heparin Sepharose column (HiTrap Heparin, manufactured by Pharmacia), a gel filtration column (Superose 6HR, manufactured by Pharmacia) or the like. It is also possible to obtain the desired DNA polymerase by a process comprising separately culturing transformants respectively containing ORF3 or ORF4 alone as described above, and subsequently mixing the cultured cells obtained, their crude extracts, or purified DNA polymerase-constituting proteins. When mixing the two kinds of DNA polymerase-constituting proteins, special procedures are not required, and the DNA polymerase possessing an activity can be obtained simply by mixing the extracts from the respective transformants or the two proteins purified therefrom in appropriate amounts.

The DNA polymerase of the present invention thus obtained provides two bands at positions corresponding to molecular weights of about 90,000 Da and about 140,000 Da on the SDS-PAGE, and these two bands corresponding to the first and second DNA polymerase-constituting proteins, respectively.

As shown in FIG. 3, the DNA polymerase of the present invention exhibits the optimum pH is in the neighborhood of 6.5 to 7.0 at 75°C in a potassium phosphate buffer. When an enzyme activity of the above DNA polymerase is assayed at various temperatures, the enzyme exhibits a high activity at 75° to 80°C. However, because the double stranded structure of the activated DNA used as a substrate for activity assay is destructed at higher temperatures, an accurate optimum temperature for the activity of the above enzyme has not been assayed. The above DNA polymerase possesses a high heat stability, retaining not less than 80% of the remaining activity even after a heat treatment at 80°C for 30 minutes, as shown in FIG. 4. This level of the heat stability allows the use of the above enzyme for PCR method. Also, when assessing the influence of aphidicolin, a specific inhibitor of α -type DNA polymerases, it is demonstrated that the activity of the above DNA polymerase is not inhibited even in the presence of 2 mM aphidicolin.

As a result of analyzing the biochemical properties of the purified DNA polymerase, the DNA polymerase of the present invention possesses very excellent primer extension activity *in vitro*. As shown in Table 1, in the case where DNA polymerase activity is assayed using a substrate in a form resulting from primer annealing to a single stranded DNA (the M13-HT Primer), higher nucleotide incorporating activity as compared to that of the activated DNA used for usual activity assaying (DNase I-treated calf thymus DNA) can be demonstrated. When the primer extension ability of the DNA polymerase of the present invention is compared with that of other DNA polymerases using the above M13-HT Primer substrate, the DNA polymerase of the present invention exhibits superior extension activity as compared to known DNA polymerases derived from *Pyrococcus furiosus* (Pfu DNA polymerase, manufactured by Stratagene) and Taq DNA polymerase derived from *Thermus aquaticus* (Taq DNA polymerase, manufactured by Takara Shuzo Co., Ltd.). Furthermore, when an activated DNA is added to this reaction system as a competitor substrate, the primer extension activities of the above two kinds of DNA polymerases are strongly inhibited, while that of the DNA polymerase of the present invention is inhibited at a low level, demonstrating that the DNA polymerase of the present invention possesses a high affinity for substrates of the primer extension type (FIG. 6).

Table 1

Substrates	Relative Activity		
	DNA Polymerase of the Present Invention	Pfu DNA Polymerase	Taq DNA Polymerase
activated DNA	100	100	100
thermal-denatured DNA	340	87	130
M13-HT primer	170	23	90
M13-RNA primer	52	0.49	38
poly dA-Oligo dT	94	390	290
poly A-Oligo dT	0.085	-	0.063

Also, the DNA polymerase of the present invention shows excellent performance when used for the PCR method. In the DNA polymerase derived from *Thermus aquaticus*, commonly used for the PCR method, it is difficult to amplify a DNA fragment of not less than 10 kbp using, the above DNA polymerase alone, and a DNA fragment of not less than 20 kbp can be amplified when used in combination with another DNA polymerase [*Proceedings of the National Academy of Sciences of the USA*, 91, 2216-2220 (1994)]. Also, the strand length of DNA amplifiable using the Pfu DNA polymerase is reportedly at most about 3 kbp. By contrast, when using the DNA polymerase of the present invention, the amplification of a DNA fragment of 20 kbp in length is made possible even when used alone without addition of any

other enzymes.

Moreover, the DNA polymerase of the present invention which also has associated 3'→5' exonuclease activity is comparable to the Pfu DNA polymerase, known to ensure very high accuracy in DNA synthesis, owing to its high activity in terms of the ratio of the exonuclease activity to the DNA polymerase activity (FIG. 5). Also, the error rate during the DNA synthesis reaction is lower for the DNA polymerase of the present invention than that of the Taq DNA polymerase. The various properties demonstrate that the DNA polymerase of the present invention serves very excellently as a reagent for genetic engineering techniques such as the PCR method.

The finding of the novel DNA polymerase genes according to the present invention also provides an interesting suggestion as follows. In order to determine the manner in which the region containing the genes for ORF3 and ORF4 encoding a novel DNA polymerase is intracellularly transcribed, the present inventors have analyzed an RNA fraction prepared from *Pyrococcus furiosus* cells by northern blotting method, RT-PCR method and primer extension method. As a result, it is confirmed that ORF1 to ORF6 are transcribed from immediately upstream of ORF1 as a single messenger RNA (mRNA). From the above finding, there is an expectation that the production of the ORF1 and the ORF2 in cells is subjected to the same control as that for the ORF3 and the ORF4. When considering in combination with the sequence homologies of ORF1, ORF2, ORF5, and ORF6 to those of CDC6 and CDC18, the CDC6 and the CDC18 being involved in the regulation for initiation of the DNA replication in yeasts, the above expectation suggests that the novel DNA polymerase of the present invention is highly likely to be a DNA polymerase important for the DNA replication. Since it is also expected that the DNA replication system of archaeobacteria, to which group *Pyrococcus furiosus* belongs, is closely related to that of eukaryotic cells, there is a possibility of the presence of an enzyme similar to the DNA polymerase of the present invention as a DNA polymerase important for replication that has not been found in eukaryotes.

It is also expected that thermostable DNA polymerases similar to the DNA polymerase of the present invention are produced in other bacteria belonging to hyperthermophilic archaeobacteria like *Pyrococcus furiosus*, including, for instance, bacteria other than *Pyrococcus furiosus* belonging to the genus *Pyrococcus*; bacteria belonging to the genus *Pyrodictium*; the genus *Thermococcus*, the genus *Staphylothermus*, and other genera. When these enzymes are constituted by two DNA polymerase-constituting proteins, like the DNA polymerase of the present invention, it is expected that a similar DNA polymerase activity is exhibited by combining one of the two DNA polymerase-constituting proteins and the DNA polymerase-constituting protein of the present invention corresponding to the other DNA polymerase-constituting protein.

The thermostable DNA polymerases similar to the DNA polymerase of the present invention, produced by the above hyperthermophilic archaeobacteria, are expected to have homology to the DNA polymerase of the present invention in terms of its amino acid sequence and the base sequence of the gene encoding thereof. It is therefore possible to obtain the gene for a thermostable DNA polymerase similar to the DNA polymerase of the present invention of which the base sequence is not identical to that of the DNA polymerase of the present invention but possesses similar enzyme activities by a process comprising introducing into an appropriate microorganism a DNA fragment obtained from one of the above thermophilic archaeobacteria by hybridization using, as a probe, a gene isolated by the present invention or a portion of the above base sequence, and assaying the DNA polymerase activity in a heat-treated lysate prepared in the same manner as the above cosmid protein library by an appropriate method.

The above hybridization can be carried out under the following conditions. Specifically, a DNA-immobilized membrane is incubated with a probe at 50°C for 12 to 20 hours in 6 x SSC, wherein 1 x SSC indicates 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0, containing 0.5% SDS, 0.1% bovine serum albumin, 0.1% polyvinyl pyrrolidone, 0.1% Ficoll 400, and 0.01% denatured salmon sperm DNA. After termination of the incubation, the membrane is washed, initiating at 37°C in 2 x SSC containing 0.5% SDS, and changing the SSC concentration to 0.1 x SSC from the starting level, while varying the SSC temperature to 50°C until the signal from the immobilized DNA becomes distinguishable from the background.

Thus, it is possible to obtain a gene for a thermostable DNA polymerase similar to the DNA polymerase of the present invention of which the DNA polymerase activity is not identical but of the same level as that of the DNA polymerase of the present invention, by introducing into an appropriate microorganism a DNA fragment obtained by a gene amplification reaction using, as a primer, a gene isolated by the present invention or a portion of the base sequence of the gene, with a DNA obtained from one of the above thermophilic archaeobacteria as a template, or a DNA fragment resulting from the thermophilic archaeobacterium by hybridization with the fragment obtained by a gene amplification reaction as a probe, and assaying the DNA polymerase activity in the same manner as above.

The present invention is hereinafter described by means of the following examples, but the scope of the present invention is not limited only to those examples. The % values shown in Examples below mean % by weight.

Example 1(1) Preparation of *Pyrococcus furiosus* Genomic DNA

5 *Pyrococcus furiosus* DSM3638 was cultured in the following manner:

A medium having a composition comprising 1% trypton, 0.5% yeast extract, 1% soluble starch, 3.5% Jamarin S Solid (Jamarin Laboratory), 0.5% Jamarin S Liquid (Jamarin Laboratory), 0.003% MgSO_4 , 0.001% NaCl , 0.0001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0001% CoSO_4 , 0.0001% $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$, 0.0001% ZnSO_4 , 0.1 ppm $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 ppm
10 $\text{KAl}(\text{SO}_4)_2$, 0.1 ppm H_3BO_3 , 0.1 ppm $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 0.25 ppm $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ was placed in a two-liter medium bottle and sterilized at 120°C for 20 minutes. After removal of dissolved oxygen by sparging with nitrogen gas thereinto, the above strain was inoculated into the resulting medium. Thereafter, the medium was cultured by kept standing at 95°C for 16 hours. After termination of the cultivation, cells were harvested by centrifugation.

The harvested cells were then suspended in 4 ml of 0.05 M Tris-HCl (pH 8.0) containing 25% sucrose. To this sus-
15 pension, 0.8 ml of lysozyme [5 mg/ml, 0.25 M Tris-HCl (pH 8.0)] and 2 ml of 0.2 M EDTA were added and incubated at 20°C for 1 hour. After adding 24 ml of an SET solution [150 mM NaCl, 1 mM EDTA, and 20 mM Tris-HCl (pH 8.0)], 4 ml of 5% SDS and 400 μl of proteinase K (10 mg/ml) were added to the resulting mixture. Thereafter, the resulting mixture was reacted at 37°C for 1 hour. After termination of the reaction, phenol-chloroform extraction and subsequent ethanol precipitation were carried out to prepare about 3.2 mg of genomic DNA.

20

(2) Preparation of Cosmid Protein Library

Four hundred micrograms of the genomic DNA from *Pyrococcus furiosus* DSM3638 was partially digested with *Sau*3A1 and fractionated by size into 35 to 50 kb fractions by density gradient ultracentrifugation method. One micro-
25 gram of the triple helix cosmid vector (manufactured by Stratagene) was digested with *Xba*I, dephosphorylated using an alkaline phosphatase (manufactured by Takara Shuzo Co., Ltd.), and further digested with *Bam*HI. The resulting treated vector was subjected to ligation after mixing with 140 μg of the above 35 to 50 kb DNA fractions. The genomic DNA fragment from *Pyrococcus furiosus* was packaged into lambda phage particles by *in vitro* packaging method using "GIGAPACK GOLD" (manufactured by Stratagene), to prepare a library. A portion of the obtained library was then trans-
30 duced into *E. coli* DH5 α MCR. Several transformants out of the resulting transformants were selected to prepare a cosmid DNA. After confirmation of the presence of an insert of appropriate size, about 500 transformants were again selected from the above library, and each was separately cultured in 150 ml of an LB medium (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl, pH 7.2) containing 100 $\mu\text{g}/\text{ml}$ ampicillin. The resulting culture was centrifuged, and the harvested cells were suspended in 1 ml of 20 mM Tris-HCl at a pH of 8.0, and the resulting suspension was then heat-treated at
35 100°C for 10 minutes. Next, ultrasonication was carried out, and a heat treatment was carried out again at 100°C for 10 minutes. The lysate obtained as a supernatant after centrifugation was used as a cosmid protein library.

(3) Assay of DNA Polymerase Activity

40 The DNA polymerase activity was assayed using calf thymus DNA (manufactured by Worthington) activated by DNase I treatment (activated DNA) as a substrate. DNA activation and assay of DNA polymerase activity were carried out by the method described in DNA Polymerase from *Escherichia coli*, 263-276 (authored by C.C. Richardson), published by Harper & Row, edited by D.R. Davis.

An assay of enzyme activity was carried out by the following method. Specifically, 50 μl of a reaction solution [20
45 mM Tris-HCl (pH 7.7), 15 mM MgCl_2 , 2 mM 2-mercaptoethanol, 0.2 mg/ml activated DNA, 40 μM each of dATP, dCTP, dGTP and dTTP, 60 nM [^3H]-dTTP (manufactured by Amersham)], containing a sample for assaying its activity, was prepared and reacted at 75°C for 15 minutes. A 40 μl portion of this reaction mixture was then spotted onto a DE paper (manufactured by Whatman) and washed with 5% Na_2HPO_4 five times. The remaining radioactivity on the DE paper was assayed using a liquid scintillation counter. The amount of enzyme which incorporated 10 nmol of [^3H]-dTTP per
50 30 minutes into the substrate DNA, assayed by the above-described enzyme activity assay method, was defined as one unit of the enzyme.

(4) Selection of Cosmid Clones Containing DNA Polymerase Gene

55 A reaction mixture comprising 20 mM Tris-HCl (pH 7.7), 2 mM MgCl_2 , 2 mM 2-mercaptoethanol, 0.2 mg/ml activated DNA, 40 μM each of dATP, dCTP, dGTP and dTTP, 60 nM [^3H]-dTTP (manufactured by Amersham) was prepared. One μl of 5 clones each of the respective extracts from the cosmid protein library, namely 5 μl of extracts as for one reaction, was added to 45 μl of this mixture. After the mixture was reacted at 75°C for 15 minutes, a 40 μl portion of

each reaction mixture was spotted onto a DE paper and washed with 5% Na₂HPO₄ five times. The remaining radioactivity on the DE paper was assayed using a liquid scintillation counter. A group found to have some activities by primary assay, wherein one group consisted of 5 clones, was separated into one clone each from the 5 clones, and then secondary assay was carried out for each clone. Since it had been already known that the cosmid DNA library included clones containing a known DNA polymerase gene by a hybridization test with the gene as a probe, designated as Clone Nos. 57, 154, 162, and 363, 5 clones possessing DNA synthesis activity other than those clones were found as Clone Nos. 41, 153, 264, 462, and 491.

(5) Preparation of Restriction Endonuclease Map

Cosmids were isolated from the above 5 clones, and each cosmid was digested with *Bam*HI. When examining the resulting migration patterns, there were demonstrated several mutually common bands, predicting that those 5 clones recombine regions with overlaps and slight shifts. With this finding in mind, the DNA inserts in Clone Nos. 264 and 491 were treated to prepare the restriction endonuclease map. The cosmids prepared from both clones were digested with various restriction endonucleases. As a result of determination for respective cleavage sites of *Kpn*I, *Not*I, *Pst*I, *Sma*I, *Xba*I, and *Xho*I (all manufactured by Takara Shuzo Co., Ltd.), digested into fragments of appropriate sizes, a map as shown in FIG. 1 was obtained.

(6) Subcloning of DNA Polymerase Gene

On the basis of the restriction endonuclease map as shown in FIG. 1, various DNA fragments of about 10 kbp in length were cut out from the cosmid derived from clone No. 264 or 491. The fragments were then subcloned into the pTV118N or pTV119N vector (manufactured by Takara Shuzo Co., Ltd.). The resulting transformant with each of the recombinant plasmids was then subjected to assaying of the thermostable DNA polymerase activity, to demonstrate that a gene for production of a highly thermostable DNA polymerase was present an *Xba*I-*Xba*I fragment of about 10 kbp. A plasmid resulting from recombination of the *Xba*I-*Xba*I fragment in the pTV118N vector was then named as plasmid pFU1001, and the *Escherichia coli* JM109 transformed with the plasmid was named as *Escherichia coli* JM109/pFU1001.

Example 2

Determination of Base Sequence of DNA Fragment Containing Novel DNA Polymerase Gene

The above *Xba*I-*Xba*I fragment, containing the DNA polymerase gene, was again cut out from the plasmid pFU1001 obtained in Example 1 with *Xba*I, and blunt-ended using a DNA blunting kit (manufactured by Takara Shuzo Co., Ltd.). The resultant was then ligated to the new pTV118N vector, previously linearized with *Sma*I, in different orientations to yield plasmids for preparing deletion mutants. The resulting plasmids were named as pFU1002 and pFU1003, respectively. Deletion mutants were sequentially prepared from both ends of the DNA insert using these plasmids. The Kilo-Sequence deletion kit (manufactured by Takara Shuzo Co., Ltd.) applying Henikoff's method (*Gene*, 28, 351-359) was used for the above preparation. The 3'-overhanging type and 5'-overhanging type restriction endonucleases used were *Pst*I and *Xba*I, respectively. The base sequence of the insert was determined by the dideoxy method using the BcaBEST dideoxy sequencing kit (manufactured by Takara Shuzo Co., Ltd.) with the various deletion mutants as templates.

An 8,450 bp sequence in the base sequence determined is shown by SEQ ID NO:5 in Sequence Listing. As a result of analysis of the base sequence, there were revealed six open reading frames (ORFs) capable of encoding proteins, present at positions corresponding to Base Nos. 123-614 (ORF1), 611-1381 (ORF2), 1384-3222 (ORF3), 3225-7013 (ORF4), 7068-7697 (ORF5), and 7711-8385 (ORF6) in the base sequence as shown by SEQ ID NO:5 in Sequence Listing. The restriction endonuclease map of the about 10 kbp *Xba*I-*Xba*I DNA fragment recombined in the plasmid pFU1001 and the location of the above-mentioned ORFs thereon are shown in FIG. 2.

In addition, the thermostable DNA polymerase activity was assayed using the above various deletion mutants. The results demonstrated that the DNA polymerase activity is lost when the deletion involves the ORF3 and ORF4 regions, regardless of whether the deletion started from upstream or downstream. This finding demonstrated that the translation products of the ORF3 and the ORF4 were important in the exhibition of the DNA polymerase activity. The base sequence of the ORF3 is shown by SEQ ID No:2 in Sequence Listing, and the amino acid sequence of the translation product of the ORF3 as deduced from the base sequence is SEQ ID NO:1 in Sequence Listing, respectively. Also, the base sequence of ORF4 is shown by SEQ ID NO:4 in Sequence Listing, and the amino acid sequence of the translation product of ORF4 as deduced from the base sequence is SEQ ID NO:3 in Sequence Listing, respectively.

Example 3Preparation of Purified DNA Polymerase Standard Preparation

5 The *Escherichia coli* JM109/pFU1001 obtained in Example 1 was cultured in 500 ml of an LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.2) containing ampicillin at a concentration of 100 µg/ml. When the culture broth turbidity reached 0.6 in A₆₀₀, an inducer, isopropyl-β-D-thiogalactoside (IPTG) was added and cultured for 16 hours. After harvesting, the harvested cells were suspended in 37 ml of a sonication buffer [50 mM Tris-HCl, pH 8.0, 0.2 mM 2-mercaptoethanol, 10% glycerol, 2.4 mM PMSF (phenylmethanesulfonyl fluoride)] and applied to an ultrasonic dis-

10 rupter. Forty-two milliliters of a crude extract was recovered as a supernatant by centrifugation at 12,000 rpm for 10 minutes, which was then heat-treated at 80°C for 15 minutes. Centrifugation was again carried out at 12,000 rpm for 10 minutes to yield 33 ml of a heat-treated enzyme solution. The above solution was then dialyzed with 800 ml of buffer A [50 mM potassium phosphate, pH 6.5, 2 mM 2-mercaptoethanol, 10% glycerol] as an external dialysis liquid for 2 hours x 4. After dialysis, 32 ml of the enzyme solution was applied to a RESOURCE Q column (manufactured by Pharmacia)

15 which was previously equilibrated with buffer A, and subjected to chromatography using an FPLC system (manufactured by Pharmacia). A development of chromatogram was carried out on a linear concentration gradient from 0 to 500 mM NaCl. A fraction having a DNA polymerase activity was eluted at 340 mM NaCl.

Ten milliliters of an enzyme solution obtained by collecting as an active fraction was desalted and concentrated by ultrafiltration, and dissolved in buffer A + 150 mM NaCl to yield 3.5 ml of an enzyme solution. The resulting enzyme solution

20 was then applied to a Hi Trap Heparin column (manufactured by Pharmacia), previously equilibrated with the same buffer. A chromatogram was developed on a linear concentration gradient from 150 to 650 mM NaCl using an FPLC system, to yield an active fraction eluted at 400 mM NaCl. Five milliliters of this fraction was concentrated to 120 µl of a solution including 50 mM potassium phosphate, pH 6.5, 2 mM 2-mercaptoethanol, and 75 mM NaCl by repeating desalting and concentration using ultrafiltration. The resulting concentrated solution was then applied to a gel filtration

25 column of Superose 6 (manufactured by Pharmacia), previously equilibrated with the same buffer, and eluted with the same buffer. As a result, a fraction having a DNA polymerase activity was eluted at positions corresponding to retention times of 34.7 minutes and 38.3 minutes. It is suggested from the results of comparison with the elution position of molecular weight markers under the same conditions that these activity peaks have molecular weights of about 385 kDa and about 220 kDa, respectively. These molecular weights corresponded to a complex formed by the translation product of ORF3 and the translation product of ORF4 in a molar ratio of 1:2 and another complex formed by the above translation products in a molar ratio of 1:1, respectively. For the former peak, however, since a possibility that a complex is formed by the two translation products in a 2:2 molar ratio cannot be negated, the molecular weight determination error increases as the molecular weight increases.

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35 Example 4(1) Biochemical Properties of DNA Polymerase

For a DNA polymerase preparation forming a complex of the translation products of ORF3 and ORF4 obtained in Example 3, namely the first DNA polymerase-constituting protein and the second DNA polymerase-constituting protein in a ratio at 1:1, optimum MgCl₂ and KCl concentrations were firstly assayed. The DNA polymerase activity was assayed in a reaction system containing 20 mM Tris-HCl, pH 7.7, 2 mM 2-mercaptoethanol, 0.2 mg/ml activated DNA, and 40 µM each of dATP, dGTP, dCTP and dTTP in the presence of 2 mM MgCl₂, while the KCl concentration was step

40 by step increased from 0 to 200 mM KCl for each 20 mM increment. As a result, the maximum activity was exhibited at a KCl concentration of 60 mM. Next, the DNA polymerase activity was assayed in the same reaction system but in the presence of 60 mM KCl in this time, while the MgCl₂ concentration was step by step increased from 0.5 to 25 mM MgCl₂ for each 2.5 mM increment, to compare at each concentration. In this case, the maximum activity was exhibited at an MgCl₂ concentration of 10 mM, and alternatively, in the absence of KCl, the maximum activity was exhibited at an MgCl₂ concentration of 17.5 mM.

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The optimum pH was then assayed. The DNA polymerase activity was assayed at 75°C by using potassium phosphate buffers at various pH levels, and preparing a reaction mixture comprising 20 mM potassium phosphate, 15 mM MgCl₂, 2 mM 2-mercaptoethanol, 0.2 mg/ml activated DNA, 40 µM each of dATP, dCTP, dGTP and dTTP, and 60 nM [³H]-dTTP. The results are shown in FIG. 3, wherein the abscissa indicates the pH, and the ordinate indicates the radioactivity incorporated in high-molecular DNA. As shown in the figure, the DNA polymerase of the present invention

50 exhibited the maximum activity at a pH of 6.5 to 7.0. When Tris-HCl was used in place of potassium phosphate, the activity increased with alkalinity, and the maximum activity was exhibited at a pH of 8.02, the highest pH level used in the assay.

55

The heat stability of the DNA polymerase of the present invention was assayed as follows: The purified DNA

polymerase was prepared to yield a mixture containing 20 mM Tris-HCl (pH 7.7), 2 mM 2-mercaptoethanol, 10% glycerol, and 0.1% bovine serum albumin, and the resulting mixture was incubated at various temperatures for 30 minutes. The remaining DNA polymerase activity was assayed. The results are shown in FIG. 4, wherein the abscissa indicates the incubation temperature, and the ordinate indicates the remaining activity. As shown in the figure, the present enzyme retained not less than 80% remaining activity even after heat treatment at 80°C for 30 minutes.

In order to compare the modes of inhibition by inhibitors, the modes of inhibition of the DNA polymerase of the present invention and an α -type DNA polymerase derived from *Pyrococcus furiosus* (Pfu DNA polymerase, manufactured by Stratagene), a known DNA polymerase, were compared using a specific inhibitor of α -type DNA polymerases, aphidicolin. The activity changes were examined, while the aphidicolin concentration was increased from 0 to 2.0 mM in the presence of 20 mM Tris-HCl, pH 7.7, 15 mM MgCl₂, 2 mM 2-mercaptoethanol, 0.2 mg/ml activated DNA, and 40 μ M each of dATP, dGTP, dCTP and dTTP. As a result, the activity of the Pfu DNA polymerase was decreased to 20% of the original activity at 1.0 mM, while the novel DNA polymerase of the present invention was not inhibited at all even at 2.0 mM.

(2) Primer Extension Reaction

Next, in order to compare the selectivity of the DNA polymerase of the present invention for different forms of substrate DNA, the following template-primer was examined. Aside from the activated DNA used for conventional assay of the activity, those prepared as substrates include a thermal-denatured DNA prepared by treating the activated DNA at 85°C for 5 minutes; M13-HT Primer prepared by annealing a 45-base synthetic deoxyribonucleotide of the sequence as shown by SEQ ID NO:6 in Sequence Listing as a primer to the M13 phage single stranded DNA (M13mp18 ssDNA, manufactured by Takara Shuzo Co., Ltd.); M13-RNA Primer prepared by annealing a 17-base synthetic ribonucleotide of the sequence as shown by SEQ ID NO:7 in Sequence Listing as a primer to the same M13 phage single stranded DNA; Poly dA-Oligo dT prepared by mixing polydeoxyadenosine (Poly dA, manufactured by Pharmacia) and oligodeoxythymidine (Oligo dT, manufactured by Pharmacia) in a 20:1 molar ratio; and Poly A-Oligo dT prepared by mixing polyadenosine (Poly A, manufactured by Pharmacia) and oligodeoxythymidine in a 20:1 molar ratio.

The DNA polymerase activity was assayed using these substrates in place of the activated DNA. The relative activity of each substrate when the activity obtained in the case of using an activated DNA as a substrate is defined as 100 is shown in Table 1. For comparison, the Pfu DNA polymerase and the Taq DNA polymerase derived from *Thermus aquaticus* (TaKaRa Taq, manufactured by Takara Shuzo Co., Ltd.) were also examined in the same manner. As shown in Table 1, in comparison with other DNA polymerases, the novel DNA polymerase of the present invention exhibited higher activity when the substrate used was the M13-HT Primer rather than the activated DNA, demonstrating that the novel DNA polymerase of the present invention is especially suitable for primer extension reaction.

The primer extension activity was further investigated extensively. The M13-HT Primer, previously labeled with [γ -³²P]-ATP (manufactured by Amersham) and T4 polynucleotide kinase (manufactured by Takara Shuzo Co., Ltd.) at the 5'-end, was used as a substrate. Ten microliters of a reaction mixture [20 mM Tris-HCl (pH 7.7), 15 mM MgCl₂, 2 mM 2-mercaptoethanol, 270 μ M each of dATP, dGTP, dCTP and dTTP] containing the above substrate in a final concentration of 0.05 μ g/ μ l and various DNA polymerases in amounts providing 0.05 units of activity as assayed with the activated DNA as a substrate was reacted at 75°C for 1, 2, 3, or 4 minutes. After termination of the reaction, 2 μ l of a reaction stop solution (95% formaldehyde, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added, subjected to thermal denaturation treatment at 95°C for 3 minutes. Two microliters of the reaction mixture was then subjected to electrophoresis using polyacrylamide gel containing 8 M urea and subsequently subjected to a preparation of autoradiogram. Also, in order to examine the extension activity in the presence of the activated DNA as a competitor substrate, the activated DNA was added to the above reaction mixture to a final concentration of 0.4 μ g/ml, and subjected to a preparation of an autoradiogram by the same procedures as described above. The autoradiogram obtained is shown in FIG. 6.

In the figure, Pol, Pfu, and Taq show the results for the DNA polymerase of the present invention, the Pfu DNA polymerase and the Taq DNA polymerase, respectively. In addition, 1, 2, 3, and 4 each indicates reaction time (min). In the figure, the representation "-" and "+" show the results obtained in the absence and in the presence, respectively, of the activated DNA. The lanes G, A, T, and C at the left end of the figure also show the results of electrophoresis of the reaction products obtained by a chain termination reaction by the dideoxy method using the same substrate as above, which were used to estimate the length of each extension product. As shown in the figure, the DNA polymerase of the present invention exhibited superior primer extension activity than those of the Pfu DNA polymerase and the Taq DNA polymerase. It was also shown that the DNA polymerase of the present invention was unlikely to be inhibited by the activated DNA, in contrast to the Taq DNA polymerase, which exhibited relatively higher primer extension activity in the absence of the activated DNA, was markedly inhibited by the addition of the activated DNA in great excess. From the above finding, it was confirmed that the DNA polymerase of the present invention possesses high affinity especially to primer extension type substrates having a form in which a single primer was annealed to a single stranded template

DNA.

(3) Presence or Absence of Associated Exonuclease Activity

5 The exonuclease activity of the DNA polymerase of the present invention was assessed as follows: As a substrate for 5'→3' exonuclease activity detection, a DNA fragment labeled with ³²P at the 5'-end was prepared by a process comprising digesting a pUC119 vector (manufactured by Takara Shuzo Co., Ltd.) with *Ssp*I (manufactured by Takara Shuzo Co., Ltd.), separating the resulting 386 bp DNA fragment by agarose gel electrophoresis, purifying the fragment, and labeling with [γ -³²P]-ATP and polynucleotide kinase. Also, as a substrate for 3'→5' exonuclease activity detection, 10 a DNA fragment labeled with ³²P at 3'-end was prepared by a process comprising digesting a pUC119 vector with *Sau*3AI, separating the resulting 341 bp DNA fragment by agarose gel electrophoresis, purifying the fragment, and carrying out a fill-in reaction using [γ -³²P]-CTP (manufactured by Amersham) and the Klenow fragment (manufactured by Takara Shuzo Co., Ltd.). The labeled DNAs were purified by gel filtration with NICK COLUMN (manufactured by Pharmacia) and used in the subsequent reaction. To a reaction solution [20 mM Tris-HCl (pH 7.7), 15 mM MgCl₂, 2 mM 2-mercaptoethanol] containing 1 ng of these labeled DNAs, 0.015 units of DNA polymerase was added, and the resulting mixture was reacted at 75°C for 2.5, 5, and 7.5 minutes. The DNAs were precipitated by adding ethanol. The radioactivity existing in the supernatant was assayed using a liquid scintillation counter, and the amount of degradation by the exonuclease activity was calculated. The DNA polymerase of the present invention was shown to possess potent 3'→5' exonuclease activity, while no 5'→3' exonuclease activity was observed. The 3'→5' exonuclease activity of the Pfu DNA polymerase, known to possess potent 3'→5' exonuclease activity, was also assayed in the same manner as above. The results are together shown in FIG. 5.

In the figure, the abscissa indicates the reaction time, and the ordinate indicates the ratio of radioactivity released into the supernatant relative to the radioactivity contained in the entire reaction mixture. Also, the open circles indicate the results for the DNA polymerase of the present invention, and the solid circles indicate those for the Pfu DNA polymerase. As shown in the figure, the DNA polymerase of the present invention showed potent 3'→5' exonuclease activity of the same level as that of the Pfu DNA polymerase, known to possess high accuracy of DNA synthesis owing to high 3'→5' exonuclease activity.

(4) Comparison of Accuracy of DNA Synthesis Reaction

30 The accuracy of DNA synthesis reaction by DNA polymerases was examined using a pUC118 vector (manufactured by Takara Shuzo Co., Ltd.), partially made single stranded (gapped duplex plasmid, as a template. The single stranded pUC118 vector was prepared by the method described in *Molecular Cloning: A Laboratory Manual*, 2nd ed., 4.44-4.48, published by Cold Spring Harbor Laboratory in 1989, edited by T. Maniatis et al., using a helper phage M13KO7 (manufactured by Takara Shuzo Co., Ltd.) with *Escherichia coli* MV1184 (manufactured by Takara Shuzo Co., Ltd.) as a host. The double stranded DNA was prepared by digesting the pUC118 vector with *Pvu*II (manufactured by Takara Shuzo Co., Ltd.), subjecting the digested vector to agarose gel electrophoresis, and recovering a DNA fragment of about 2.8 kbp.

One microgram of the above single stranded DNA and 2 μ g of the double stranded DNA were mixed to make 180 μ l of a mixture with sterile distilled water, and the solution was then incubated at 70°C for 10 minutes. Thereafter, twenty microliters of 20 x SSC was added to the resulting mixture, and the mixture was further kept standing at 60°C for 10 minutes. The DNA was recovered by subjecting to ethanol precipitation. A portion thereof was subjected to agarose gel electrophoresis, and it was confirmed that a gapped duplex plasmid was obtained. Thirty microliters of a reaction mixture [10 mM Tris-HCl, pH 8.5, 50 mM KCl, 10 mM MgCl₂, 1 mM each of dATP, dCTP, dGTP and dTTP], containing an amount one-tenth that of the resulting gapped duplex plasmid was incubated at 70°C for 3 minutes, after which 0.5 units of DNA polymerase was added thereto, and a DNA synthesis reaction was carried out at 70°C for 10 minutes. After termination of the reaction, *Escherichia coli* DH5 α (manufactured by BRL) was transformed using 10 μ l of the reaction mixture. The resulting transformant was cultured at 37°C for 18 hours on an LB plate containing 100 μ g/ml ampicillin, 0.1 mM IPTG, and 40 μ g/ml 5-bromo-4-chloro-3-indolyl- β -D-galactoside. The white or blue colonies formed on the plate were counted, and the formation rate of white colonies which were resulted from a DNA synthesis error was calculated. As a result, the white colony formation rate (%) was 3.18% when the Taq DNA polymerase was used as the DNA polymerase, in contrast to a lower formation rate of 1.61% when the DNA polymerase of the present invention was used.

(5) Application to PCR

In order to compare the performance of the DNA polymerase of the present invention in PCR with that of the Taq DNA polymerase, PCR was carried out with λ -DNA as a template. The reaction mixture for the DNA polymerase of the

present invention had the following composition: 10 mM Tris-HCl (pH 9.2), 3.5 mM MgCl₂, 75 mM KCl, 400 μM each of dATP, dCTP, dGTP and dTTP, 0.01% bovine serum albumin (BSA), and 0.1% Triton X-100. The reaction solution for the Taq DNA polymerase had the following composition: 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, and 400 μM each of dATP, dCTP, dGTP and dTTP. Fifty microliters of a reaction mixture containing 5.0 ng/50 μl λ-DNA (manufactured by Takara Shuzo Co., Ltd.), 10 pmol/50 μl each of primer λ1 and primer λ11, and 3.7 units/50 μl DNA polymerase was prepared. The base sequences of the primer λ1 and the primer λ11 are shown by SEQ ID NO:8 and SEQ ID NO:9 in Sequence Listing, respectively. After, a 30-cycle PCR was carried out with the above reaction mixture, wherein one cycle is defined at 98°C for 10 seconds and at 68°C for 10 seconds. Five microliters of the reaction mixture was subjected to agarose gel electrophoresis, and the amplified DNA fragment was confirmed by staining with ethidium bromide. As a result, it was demonstrated that the DNA fragment amplification was not found when the Taq DNA polymerase was used, in contrast to the DNA polymerase of the present invention where amplification of a DNA fragment of about 20 kbp was confirmed.

The experiment was then carried out by changing the primer to the primer λ1 and the primer λ10. The base sequence of the primer λ10 is shown by SEQ ID NO:10 in Sequence Listing. Twenty-five microliters of a reaction mixture having a similar composition to that shown above and containing 2.5 ng of λ-DNA, 10 pmol of the primer λ1 and the primer λ10, respectively, and 3.7 units of DNA polymerase was prepared. The reaction mixture was reacted in 5 cycles under the same reaction conditions as those described above, and 5 μl of the reaction mixture was subjected to agarose gel electrophoresis and stained with ethidium bromide. It was demonstrated that no specific amplification was observed when the Taq DNA polymerase was used, in contrast to the DNA polymerase of the present invention where a DNA fragment of about 15 kbp was amplified.

Example 5

(1) Construction of Plasmid for Expression of ORF3 Translation Product Alone

PCR was carried out using a mutant plasmid 6-82 as a template, the mutant plasmid being prepared by deleting the portion immediately downstream of the ORF3 from the DNA insert in the plasmid pFU1002 described in Example 2, wherein the ORF1 to the ORF6 were located downstream of the *lac* promoter on the vector and also using a primer M4 (manufactured by Takara Shuzo Co., Ltd) and the primer NO3 whose base sequence is shown by SEQ ID:11 in Sequence Listing. The DNA polymerase used for the PCR was the Pfu DNA polymerase (manufactured by Stratagene), which possessed high accuracy of synthesis reaction. A 25-cycle reaction of 100 μl of a reaction mixture for PCR [20 mM Tris-HCl, pH 8.2, 10 mM KCl, 20 mM MgCl₂, 6 mM (NH₄)₂SO₄, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1% Triton X-100, 0.01% BSA] containing 1 ng of a template DNA, 25 pmol of each primer, and 2.5 units of the Pfu DNA polymerase was carried out, wherein one cycle is defined as at 94°C for 0.5 minutes, at 55°C for 0.5 minutes and at 72°C for 2 minutes. The amplified DNA fragment of about 2 kbp was digested with *Nco*I and *Sph*I (each manufactured by Takara Shuzo Co., Ltd.) and inserted into between the *Nco*I-*Sph*I sites of the pTV118N vector (manufactured by Takara Shuzo Co., Ltd.) to prepare a plasmid pFU-ORF3. The DNA insert in the above plasmid contains ORF3 alone in translatable conditions.

(2) Construction of Plasmid for Expression of ORF4 Translation Product Alone

PCR was carried out using a mutant plasmid 6-2 as a template, the mutant plasmid being prepared by deleting the portion downstream of the center portion of the ORF4 from the DNA insert in the above-described plasmid pFU1002, the primer M4, and the primer NO4 of which the base sequence is shown by SEQ ID NO:12 in Sequence Listing. The reaction was carried out under the same conditions as those for Example 5-(1) described above, except that the template DNA was replaced with the plasmid 6-2, and the primer NO3 was replaced with the primer NO4. A DNA fragment of about 1.6 kbp obtained by digesting the above amplified DNA fragment with *Nco*I and *Nhe*I (manufactured by Takara Shuzo Co., Ltd.), together with an about 3.3 kbp *Nhe*I-*Sal*I fragment, including the latter portion of ORF4, isolated from the above plasmid pFU1002 was inserted between the *Nco*I-*Xho*I sites of a pET15b vector (manufactured by Novagen) to prepare a plasmid pFU-ORF4. The DNA insert in the plasmid contains ORF4 alone in translatable conditions.

(3) Reconstitution of DNA Polymerase with ORF3 and ORF4 Translation Products

The *Escherichia coli* JM109 transformed with the above-described plasmid pFU-ORF3, *Escherichia coli* JM109/pFU-ORF3, and the *Escherichia coli* HMS174 transformed with the above-described plasmid pFU-ORF4, *Escherichia coli* HMS174/pFU-ORF4, were separately cultured, and then the translation products of the two ORFs expressed in their cells were purified. The cultivation of the transformants and the preparation of the crude extracts were carried out by the methods described in Example 3. Purification of both translation products was carried out using col-

umns such as RESOURCE Q, HiTrap Heparin, and Superose 6, while the behaviors of the translation products on SDS-PAGE were monitored. It was confirmed that although neither of the ORF translation products thus purified exhibited the DNA polymerase activity when assayed alone, thermostable DNA polymerase activity was exhibited when they were mixed together.

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INDUSTRIAL APPLICABILITY

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The present invention can provide a novel DNA polymerase possessing both high primer extensibility and high 3'→5' exonuclease activity. The enzyme is suitable for its use for PCR method, which is useful for a reagent for genetic engineering investigation. It is also possible to produce the enzyme by genetic engineering using the genes encoding the DNA polymerase of the present invention.

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SEQUENCE LISTING

5 SEQ ID NO:1
 SEQUENCE LENGTH: 613
 SEQUENCE TYPE: amino acid
 10 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULAR TYPE: peptide
 SEQUENCE DESCRIPTION:
 15 Met Asp Glu Phe Val Lys Ser Leu Leu Lys Ala Asn Tyr Leu Ile
 5 10 15
 Thr Pro Ser Ala Tyr Tyr Leu Leu Arg Glu Tyr Tyr Glu Lys Gly
 20 20 25 30
 Glu Phe Ser Ile Val Glu Leu Val Lys Phe Ala Arg Ser Arg Glu
 35 40 45
 25 Ser Tyr Ile Ile Thr Asp Ala Leu Ala Thr Glu Phe Leu Lys Val
 50 55 60
 Lys Gly Leu Glu Pro Ile Leu Pro Val Glu Thr Lys Gly Gly Phe
 65 70 75
 30 Val Ser Thr Gly Glu Ser Gln Lys Glu Gln Ser Tyr Glu Glu Ser
 80 85 90
 Phe Gly Thr Lys Glu Glu Ile Ser Gln Glu Ile Lys Glu Gly Glu
 35 95 100 105
 Ser Phe Ile Ser Thr Gly Ser Glu Pro Leu Glu Glu Glu Leu Asn
 110 115 120
 40 Ser Ile Gly Ile Glu Glu Ile Gly Ala Asn Glu Glu Leu Val Ser
 125 130 135
 Asn Gly Asn Asp Asn Gly Gly Glu Ala Ile Val Phe Asp Lys Tyr
 140 145 150
 45 Gly Tyr Pro Met Val Tyr Ala Pro Glu Glu Ile Glu Val Glu Glu
 155 160 165
 Lys Glu Tyr Ser Lys Tyr Glu Asp Leu Thr Ile Pro Met Asn Pro
 50 170 175 180
 Asp Phe Asn Tyr Val Glu Ile Lys Glu Asp Tyr Asp Val Val Phe

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		185		190		195
	Asp Val Arg Asn	Val Lys Leu Lys Pro	Pro Lys Val Lys Asn Gly			
5		200		205		210
	Asn Gly Lys Glu	Gly Glu Ile Ile Val	Glu Ala Tyr Ala Ser Leu			
		215		220		225
10	Phe Arg Ser Arg	Leu Lys Lys Leu Arg	Lys Ile Leu Arg Glu Asn			
		230		235		240
	Pro Glu Leu Asp	Asn Val Val Asp Ile	Gly Lys Leu Lys Tyr Val			
		245		250		255
15	Lys Glu Asp Glu	Thr Val Thr Ile Ile	Gly Leu Val Asn Ser Lys			
		260		265		270
	Arg Glu Val Asn	Lys Gly Leu Ile Phe	Glu Ile Glu Asp Leu Thr			
20		275		280		285
	Gly Lys Val Lys	Val Phe Leu Pro Lys	Asp Ser Glu Asp Tyr Arg			
		290		295		300
25	Glu Ala Phe Lys	Val Leu Pro Asp Ala	Val Val Ala Phe Lys Gly			
		305		310		315
	Val Tyr Ser Lys	Arg Gly Ile Leu Tyr	Ala Asn Lys Phe Tyr Leu			
		320		325		330
30	Pro Asp Val Pro	Leu Tyr Arg Arg Gln	Lys Pro Pro Leu Glu Glu			
		335		340		345
	Lys Val Tyr Ala	Ile Leu Ile Ser Asp	Ile His Val Gly Ser Lys			
35		350		355		360
	Glu Phe Cys Glu	Asn Ala Phe Ile Lys	Phe Leu Glu Trp Leu Asn			
		365		370		375
40	Gly Asn Val Glu	Thr Lys Glu Glu Glu	Glu Ile Val Ser Arg Val			
		380		385		390
	Lys Tyr Leu Ile	Ile Ala Gly Asp Val	Val Asp Gly Val Gly Val			
		395		400		405
45	Tyr Pro Gly Gln	Tyr Ala Asp Leu Thr	Ile Pro Asp Ile Phe Asp			
		410		415		420
	Gln Tyr Glu Ala	Leu Ala Asn Leu Leu	Ser His Val Pro Lys His			
50		425		430		435
	Ile Thr Met Phe	Ile Ala Pro Gly Asn	His Asp Ala Ala Arg Gln			

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	440	445	450
	Ala Ile Pro Gln Pro Glu Phe Tyr Lys	Glu Tyr Ala Lys Pro Ile	
5	455	460	465
	Tyr Lys Leu Lys Asn Ala Val Ile Ile	Ser Asn Pro Ala Val Ile	
	470	475	480
	Arg Leu His Gly Arg Asp Phe Leu Ile	Ala His Gly Arg Gly Ile	
10	485	490	495
	Glu Asp Val Val Gly Ser Val Pro Gly	Leu Thr His His Lys Pro	
	500	505	510
	Gly Leu Pro Met Val Glu Leu Leu Lys	Met Arg His Val Ala Pro	
15	515	520	525
	Met Phe Gly Gly Lys Val Pro Ile Ala	Pro Asp Pro Glu Asp Leu	
	530	535	540
20	Leu Val Ile Glu Glu Val Pro Asp Val	Val His Met Gly His Val	
	545	550	555
	His Val Tyr Asp Ala Val Val Tyr Arg	Gly Val Gln Leu Val Asn	
	560	565	570
25	Ser Ala Thr Trp Gln Ala Gln Thr Glu	Phe Gln Lys Met Val Asn	
	575	580	585
	Ile Val Pro Thr Pro Ala Lys Val Pro	Val Val Asp Ile Asp Thr	
	590	595	600
30	Ala Lys Val Val Lys Val Leu Asp Phe	Ser Gly Trp Cys	
	605	610	

35 SEQ ID NO:2
 SEQUENCE LENGTH: 1839
 SEQUENCE TYPE: nucleic acid
 STRANDEDNESS: double
 40 TOPOLOGY: linear
 MOLECULAR TYPE: Genomic DNA
 SEQUENCE DESCRIPTION:
 ATGGATGAAT TTGTAAATC ACTTCTAAAA GCTAACTATC TAATAACTCC CTCTGCCTAC 60
 45 TATCTCTTGA GAGAATACTA TGAAAAAGGT GAATTCTCAA TTGTGGAGCT GGTAATAATT 120
 GCAAGATCAA GAGAGAGCTA CATAATTACT GATGCTTTAG CAACAGAATT CCTTAAAGTT 180

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AAAGGCCTTG AACCAATTCT TCCAGTGGAA ACAAAGGGGG GTTTTGTTTC CACTGGAGAG 240
TCCCAAAAAG AGCAGTCTTA TGAAGAGTCT TTTGGGACTA AAGAAGAAAT TTCCCAGGAG 300
5 ATTAAGAAG GAGAGAGTTT TATTTCCACT GGAAGTGAAC CACTTGAAGA GGAGCTCAAT 360
AGCATTGGAA TTGAGGAAAT TGGGGCAAAT GAAGAGTTAG TTTCTAATGG AAATGACAAT 420
GGTGGAGAGG CAATTGTCTT TGACAAATAT GGCTATCCAA TGGTATATGC TCCAGAAGAA 480
ATAGAGGTTG AGGAGAAGGA GTACTCGAAG TATGAAGATC TGACAATACC CATGAACCCC 540
10 GACTTCAATT ATGTGGAAAT AAAGGAAGAT TATGATGTTG TCTTCGATGT TAGGAATGTA 600
AAGCTGAAGC CTCCTAAGGT AAAGAACGGT AATGGGAAGG AAGGTGAAAT AATTGTTGAA 660
GCTTATGCTT CTCTCTTCAG GAGTAGGTTG AAGAAGTTAA GGAAAATACT AAGGGAAAAT 720
CCTGAATTGG ACAATGTTGT TGATATTGGG AAGCTGAAGT ATGTGAAGGA AGATGAAACC 780
15 GTGACAATAA TAGGGCTTGT CAATTCCAAG AGGGAAGTGA ATAAAGGATT GATATTTGAA 840
ATAGAAGATC TCACAGGAAA GGTTAAAGTT TTCTGCCGA AAGATTCGGA AGATTATAGG 900
GAGGCATTTA AGGTTCTTCC AGATGCCGTC GTCGCTTTTA AGGGGGTGTA TTCAAAGAGG 960
20 GGAATTTTGT ACGCCAACAA GTTTTACCTT CCAGACGTTT CCCTCTATAG GAGACAAAAG 1020
CCTCCACTGG AAGAGAAAGT TTATGCTATT CTCATAAGTG ATATACACGT CGGAAGTAAA 1080
GAGTTCTGCG AAAATGCCTT CATAAAGTTC TTAGAGTGGC TCAATGAAAA CGTTGAAACT 1140
AAGGAAGAGG AAGAAATCGT GAGTAGGGTT AAGTATCTAA TCATTGCAGG AGATGTTGTT 1200
25 GATGGTGTG GCGTTTATCC GGGCCAGTAT GCCGACTTGA CGATTCCAGA TATATTCGAC 1260
CAGTATGAGG CCCTCGCAA CTTTCTCTCT CACGTTCTTA AGCACATAAC AATGTTTCATT 1320
GCCCCAGGAA ACCACGATGC TGCTAGGCAA GCTATTCCCC AACCAGAATT CTACAAAGAG 1380
TATGCAAAAC CTATATACAA GCTCAAGAAC GCCGTGATAA TAAGCAATCC TGCTGTAATA 1440
30 AGACTACATG GTAGGGACTT TCTGATAGCT CATGGTAGGG GGATAGAGGA TGTCGTTGGA 1500
AGTGTTCCTG GGTGACCCA TCACAAGCCC GGCCTCCCAA TGGTTGAACT ATTGAAGATG 1560
AGGCATGTAG CTCCAATGTT TGGAGGAAAG GTTCCAATAG CTCCTGATCC AGAAGATTTG 1620
CTTGTTATAG AAGAAGTTCC TGATGTAGTT CACATGGGTC ACGTTCACGT TTACGATGCG 1680
35 GTAGTTTATA GGGGAGTTCA GCTGGTTAAC TCCGCCACCT GGCAGGCTCA GACCGAGTTC 1740
CAGAAGATGG TGAACATAGT TCCAACGCCT GCAAAGGTTT CCGTTGTTGA TATTGATACT 1800
GCAAAAGTTG TCAAGGTTTT GGACTTTAGT GGGTGGTGC 1839

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SEQ ID NO:3

SEQUENCE LENGTH: 1263

SEQUENCE TYPE: amino acid

45

STRANDEDNESS: single

TOPOLOGY: linear

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MOLECULAR TYPE: peptide

SEQUENCE DESCRIPTION:

5	Met	Glu	Leu	Pro	Lys	Glu	Ile	Glu	Glu	Tyr	Phe	Glu	Met	Leu	Gln	
					5					10					15	
	Arg	Glu	Ile	Asp	Lys	Ala	Tyr	Glu	Ile	Ala	Lys	Lys	Ala	Arg	Ser	
10					20					25					30	
	Gln	Gly	Lys	Asp	Pro	Ser	Thr	Asp	Val	Glu	Ile	Pro	Gln	Ala	Thr	
					35					40					45	
	Asp	Met	Ala	Gly	Arg	Val	Glu	Ser	Leu	Val	Gly	Pro	Pro	Gly	Val	
15					50					55					60	
	Ala	Gln	Arg	Ile	Arg	Glu	Leu	Leu	Lys	Glu	Tyr	Asp	Lys	Glu	Ile	
					65					70					75	
20	Val	Ala	Leu	Lys	Ile	Val	Asp	Glu	Ile	Ile	Glu	Gly	Lys	Phe	Gly	
					80					85					90	
	Asp	Phe	Gly	Ser	Lys	Glu	Lys	Tyr	Ala	Glu	Gln	Ala	Val	Arg	Thr	
25					95					100					105	
	Ala	Leu	Ala	Ile	Leu	Thr	Glu	Gly	Ile	Val	Ser	Ala	Pro	Leu	Glu	
					110					115					120	
30	Gly	Ile	Ala	Asp	Val	Lys	Ile	Lys	Arg	Asn	Thr	Trp	Ala	Asp	Asn	
					125					130					135	
	Ser	Glu	Tyr	Leu	Ala	Leu	Tyr	Tyr	Ala	Gly	Pro	Ile	Arg	Ser	Ser	
					140					145					150	
35	Gly	Gly	Thr	Ala	Gln	Ala	Leu	Ser	Val	Leu	Val	Gly	Asp	Tyr	Val	
					155					160					165	
	Arg	Arg	Lys	Leu	Gly	Leu	Asp	Arg	Phe	Lys	Pro	Ser	Gly	Lys	His	
40					170					175					180	
	Ile	Glu	Arg	Met	Val	Glu	Glu	Val	Asp	Leu	Tyr	His	Arg	Ala	Val	
					185					190					195	
45	Ser	Arg	Leu	Gln	Tyr	His	Pro	Ser	Pro	Asp	Glu	Val	Arg	Leu	Ala	
					200					205					210	
	Met	Arg	Asn	Ile	Pro	Ile	Glu	Ile	Thr	Gly	Glu	Ala	Thr	Asp	Asp	
					215					220					225	
50	Val	Glu	Val	Ser	His	Arg	Asp	Val	Glu	Gly	Val	Glu	Thr	Asn	Gln	
					230					235					240	

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	Leu Arg Gly Gly	Ala Ile Leu Val	Leu Ala Glu Gly	Val Leu Gln	
		245	250		255
5	Lys Ala Lys Lys	Leu Val Lys Tyr Ile	Asp Lys Met Gly	Ile Asp	
		260	265		270
	Gly Trp Glu Trp	Leu Lys Glu Phe Val	Glu Ala Lys Glu	Lys Gly	
10		275	280		285
	Glu Glu Ile Glu	Glu Ser Glu Ser Lys	Ala Glu Glu Ser	Lys Val	
		290	295		300
15	Glu Thr Arg Val	Glu Val Glu Lys Gly	Phe Tyr Tyr Lys	Leu Tyr	
		305	310		315
	Glu Lys Phe Arg	Ala Glu Ile Ala Pro	Ser Glu Lys Tyr	Ala Lys	
		320	325		330
20	Glu Ile Ile Gly	Gly Arg Pro Leu Phe	Ala Gly Pro Ser	Glu Asn	
		335	340		345
	Gly Gly Phe Arg	Leu Arg Tyr Gly Arg	Ser Arg Val Ser	Gly Phe	
25		350	355		360
	Ala Thr Trp Ser	Ile Asn Pro Ala Thr	Met Val Leu Val	Asp Glu	
		365	370		375
30	Phe Leu Ala Ile	Gly Thr Gln Met Lys	Thr Glu Arg Pro	Gly Lys	
		380	385		390
	Gly Ala Val Val	Thr Pro Ala Thr Thr	Ala Glu Gly Pro	Ile Val	
		395	400		405
35	Lys Leu Lys Asp	Gly Ser Val Val Arg	Val Asp Asp Tyr	Asn Leu	
		410	415		420
	Ala Leu Lys Ile	Arg Asp Glu Val Glu	Glu Ile Leu Tyr	Leu Gly	
40		425	430		435
	Asp Ala Ile Ile	Ala Phe Gly Asp Phe	Val Glu Asn Asn	Gln Thr	
		440	445		450
45	Leu Leu Pro Ala	Asn Tyr Val Glu Glu	Trp Trp Ile Gln	Glu Phe	
		455	460		465
	Val Lys Ala Val	Asn Glu Ala Tyr Glu	Val Glu Leu Arg	Pro Phe	
		470	475		480
50	Glu Glu Asn Pro	Arg Glu Ser Val Glu	Glu Ala Ala Glu	Tyr Leu	
		485	490		495

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	Glu Val Asp Pro Glu Phe Leu Ala Lys Met Leu Tyr Asp Pro Leu	
	500	505 510
5	Arg Val Lys Pro Pro Val Glu Leu Ala Ile His Phe Ser Glu Ile	
	515	520 525
	Leu Glu Ile Pro Leu His Pro Tyr Tyr Thr Leu Tyr Trp Asn Thr	
10	530	535 540
	Val Asn Pro Lys Asp Val Glu Arg Leu Trp Gly Val Leu Lys Asp	
	545	550 555
	Lys Ala Thr Ile Glu Trp Gly Thr Phe Arg Gly Ile Lys Phe Ala	
15	560	565 570
	Lys Lys Ile Glu Ile Ser Leu Asp Asp Leu Gly Ser Leu Lys Arg	
	575	580 585
20	Thr Leu Glu Leu Leu Gly Leu Pro His Thr Val Arg Glu Gly Ile	
	590	595 600
	Val Val Val Asp Tyr Pro Trp Ser Ala Ala Leu Leu Thr Pro Leu	
25	605	610 615
	Gly Asn Leu Glu Trp Glu Phe Lys Ala Lys Pro Phe Tyr Thr Val	
	620	625 630
	Ile Asp Ile Ile Asn Glu Asn Asn Gln Ile Lys Leu Arg Asp Arg	
30	635	640 645
	Gly Ile Ser Trp Ile Gly Ala Arg Met Gly Arg Pro Glu Lys Ala	
	650	655 660
35	Lys Glu Arg Lys Met Lys Pro Pro Val Gln Val Leu Phe Pro Ile	
	665	670 675
	Gly Leu Ala Gly Gly Ser Ser Arg Asp Ile Lys Lys Ala Ala Glu	
40	680	685 690
	Glu Gly Lys Ile Ala Glu Val Glu Ile Ala Phe Phe Lys Cys Pro	
	695	700 705
	Lys Cys Gly His Val Gly Pro Glu Thr Leu Cys Pro Glu Cys Gly	
45	710	715 720
	Ile Arg Lys Glu Leu Ile Trp Thr Cys Pro Lys Cys Gly Ala Glu	
	725	730 735
50	Tyr Thr Asn Ser Gln Ala Glu Gly Tyr Ser Tyr Ser Cys Pro Lys	
	740	745 750

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	Cys	Asn	Val	Lys	Leu	Lys	Pro	Phe	Thr	Lys	Arg	Lys	Ile	Lys	Pro	
					755					760					765	
5	Ser	Glu	Leu	Leu	Asn	Arg	Ala	Met	Glu	Asn	Val	Lys	Val	Tyr	Gly	
					770					775					780	
	Val	Asp	Lys	Leu	Lys	Gly	Val	Met	Gly	Met	Thr	Ser	Gly	Trp	Lys	
10					785					790					795	
	Ile	Ala	Glu	Pro	Leu	Glu	Lys	Gly	Leu	Leu	Arg	Ala	Lys	Asn	Glu	
					800					805					810	
	Val	Tyr	Val	Phe	Lys	Asp	Gly	Thr	Ile	Arg	Phe	Asp	Ala	Thr	Asp	
15					815					820					825	
	Ala	Pro	Ile	Thr	His	Phe	Arg	Pro	Arg	Glu	Ile	Gly	Val	Ser	Val	
					830					835					840	
20	Glu	Lys	Leu	Arg	Glu	Leu	Gly	Tyr	Thr	His	Asp	Phe	Glu	Gly	Lys	
					845					850					855	
	Pro	Leu	Val	Ser	Glu	Asp	Gln	Ile	Val	Glu	Leu	Lys	Pro	Gln	Asp	
25					860					865					870	
	Val	Ile	Leu	Ser	Lys	Glu	Ala	Gly	Lys	Tyr	Leu	Leu	Arg	Val	Ala	
					875					880					885	
	Arg	Phe	Val	Asp	Asp	Leu	Leu	Glu	Lys	Phe	Tyr	Gly	Leu	Pro	Arg	
30					890					895					900	
	Phe	Tyr	Asn	Ala	Glu	Lys	Met	Glu	Asp	Leu	Ile	Gly	His	Leu	Val	
					905					910					915	
35	Ile	Gly	Leu	Ala	Pro	His	Thr	Ser	Ala	Gly	Ile	Val	Gly	Arg	Ile	
					920					925					930	
	Ile	Gly	Phe	Val	Asp	Ala	Leu	Val	Gly	Tyr	Ala	His	Pro	Tyr	Phe	
40					935					940					945	
	His	Ala	Ala	Lys	Arg	Arg	Asn	Cys	Asp	Gly	Asp	Glu	Asp	Ser	Val	
					950					955					960	
45	Met	Leu	Leu	Leu	Asp	Ala	Leu	Leu	Asn	Phe	Ser	Arg	Tyr	Tyr	Leu	
					965					970					975	
	Pro	Glu	Lys	Arg	Gly	Gly	Lys	Met	Asp	Ala	Pro	Leu	Val	Ile	Thr	
					980					985					990	
50	Thr	Arg	Leu	Asp	Pro	Arg	Glu	Val	Asp	Ser	Glu	Val	His	Asn	Met	
					995					1000					1005	

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	Asp Val Val Arg Tyr Tyr Pro Leu Glu Phe Tyr Glu Ala Thr Tyr		
	1010	1015	1020
5	Glu Leu Lys Ser Pro Lys Glu Leu Val Arg Val Ile Glu Gly Val		
	1025	1030	1035
	Glu Asp Arg Leu Gly Lys Pro Glu Met Tyr Tyr Gly Ile Lys Phe		
10	1040	1045	1050
	Thr His Asp Thr Asp Asp Ile Ala Leu Gly Pro Lys Met Ser Leu		
	1055	1060	1065
	Tyr Lys Gln Leu Gly Asp Met Glu Glu Lys Val Lys Arg Gln Leu		
15	1070	1075	1080
	Thr Leu Ala Glu Arg Ile Arg Ala Val Asp Gln His Tyr Val Ala		
	1085	1090	1095
20	Glu Thr Ile Leu Asn Ser His Leu Ile Pro Asp Leu Arg Gly Asn		
	1100	1105	1110
	Leu Arg Ser Phe Thr Arg Gln Glu Phe Arg Cys Val Lys Cys Asn		
25	1115	1120	1125
	Thr Lys Tyr Arg Arg Pro Pro Leu Asp Gly Lys Cys Pro Val Cys		
	1130	1135	1140
30	Gly Gly Lys Ile Val Leu Thr Val Ser Lys Gly Ala Ile Glu Lys		
	1145	1150	1155
	Tyr Leu Gly Thr Ala Lys Met Leu Val Ala Asn Tyr Asn Val Lys		
	1160	1165	1170
35	Pro Tyr Thr Arg Gln Arg Ile Cys Leu Thr Glu Lys Asp Ile Asp		
	1175	1180	1185
	Ser Leu Phe Glu Tyr Leu Phe Pro Glu Ala Gln Leu Thr Leu Ile		
40	1190	1195	1200
	Val Asp Pro Asn Asp Ile Cys Met Lys Met Ile Lys Glu Arg Thr		
	1205	1210	1215
45	Gly Glu Thr Val Gln Gly Gly Leu Leu Glu Asn Phe Asn Ser Ser		
	1220	1225	1230
	Gly Asn Asn Gly Lys Lys Ile Glu Lys Lys Glu Lys Lys Ala Lys		
	1235	1240	1245
50	Glu Lys Pro Lys Lys Lys Lys Val Ile Ser Leu Asp Asp Phe Phe		
	1250	1255	1260

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Ser Lys Arg

5 SEQ ID NO:4
SEQUENCE LENGTH: 3789
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: double
10 TOPOLOGY: linear
MOLECULAR TYPE: Genomic DNA
SEQUENCE DESCRIPTION:
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GCTTACGAGA TTGCTAAGAA GGCTAGGAGT CAGGGTAAAG ACCCCTCAAC CGATGTTGAG 120
ATTCCCCAGG CTACAGACAT GGCTGGAAGA GTTGAAGAGCT TAGTTGGCCC TCCCGGAGTT 180
GCTCAGAGAA TTAGGGAGCT TTTAAAAGAG TATGATAAGG AAATTGTTGC TTAAAGATA 240
20 GTTGATGAGA TAATTGAGGG CAAATTTGGT GATTTTGGAA GTAAAGAGAA GTACGCTGAA 300
CAGGCTGTAA GGACAGCCTT GGCAATATTA ACTGAGGGTA TTGTTTCTGC TCCACTTGAG 360
GGTATAGCTG ATGTTAAAAT CAAGCGAAAC ACCTGGGCTG ATAACCTCTGA ATACCTCGCC 420
CTTTACTATG CTGGGCCAAT TAGGAGTTCT GGTGGAAGCTG CTCAAGCTCT CAGTGTACTT 480
25 GTTGGTGATT ACGTTAGGCG AAAGCTTGGC CTTGATAGGT TTAAGCCAAG TGGGAAGCAT 540
ATAGAGAGAA TGTTGAGGA AGTTGACCTC TATCATAGAG CTGTTTCAAG GCTTCAATAT 600
CATCCCTCAC CTGATGAAGT GAGATTAGCA ATGAGGAATA TTCCCATAGA AATCACTGGT 660
GAAGCCACTG ACGATGTGGA GGTTCCTCAT AGAGATGTAG AGGGAGTTGA GACAAATCAG 720
30 CTGAGAGGAG GAGCGATCCT AGTTTGGCG GAGGGTGTTT TCCAGAAGGC TAAAAAGCTC 780
GTGAAATACA TTGACAAGAT GGGGATTGAT GGATGGGAGT GGCTTAAAGA GTTTGTAGAG 840
GCTAAAGAAA AAGGTGAAGA AATCGAAGAG AGTGAAAGTA AAGCCGAGGA GTCAAAAGTT 900
GAAACAAGGG TGGAGGTAGA GAAGGGATTC TACTACAAGC TCTATGAGAA ATTTAGGGCT 960
35 GAGATTGCCC CAAGCGAAAA GTATGCAAAG GAAATAATTG GTGGGAGGCC GTTATTCGCT 1020
GGACCCTCGG AAAATGGGGG ATTTAGGCTT AGATATGGTA GAAGTAGGGT GAGTGGATTT 1080
GCAACATGGA GCATAAATCC AGCAACAATG GTTTGGTTG ACGAGTTCTT GGCCATTGGA 1140
40 ACTCAAATGA AAACCGAGAG GCCTGGGAAA GGTGCAGTAG TGAATCCAGC AACAACCGCT 1200
GAAGGGCCGA TTGTTAAGCT AAAGGATGGG AGTGTTGTTA GGTTGATGA TTACAACTTG 1260
GCCCTCAAAA TAAGGGATGA AGTCGAAGAG ATACTTTATT TGGGAGATGC AATCATAGCC 1320
TTTGGAGACT TTGTGGAGAA CAATCAAACCT CTCCTTCCTG CAACTATGT AGAGGAGTGG 1380
45 TGGATCCAAG AGTTCGTAAA GGCCGTTAAT GAGGCATATG AAGTTGAGCT TAGACCCTTT 1440
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 5 GTAAATCCTA AAGATGTTGA AAGACTTTGG GGAGTATTAA AAGACAAGGC CACCATAGAA 1680
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 10 GAGTTTAAGG CCAAGCCCTT CTACACTGTA ATAGACATCA TTAACGAGAA CAATCAGATA 1920
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 15 TTCTTCAAGT GTCCGAAGTG TGGCCATGTA GGGCCTGAAA CTCTCTGTCC CGAGTGTGGG 2160
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 20 AGGAAGATAA AGCCCTCAGA GCTCTTAAAC AGGGCCATGG AAAACGTGAA GGTTTATGGA 2340
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 AAGCCTGAAA TGTATTACGG AATAAAGTTC ACCCAGGATA CCGACGACAT AGCTCTAGGA 3180
 40 CCAAAGATGA GCCTCTACAA GCAGTTGGGA GATATGGAGG AGAAAGTGAA GAGGCAATTG 3240
 ACATTGGCAG AGAGAATTAG AGCTGTGGAT CAACACTATG TTGCTGAAAC AATCCTCAAC 3300
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 TGTGTGAAGT GTAACACAAA GTACAGAAGG CCGCCCTTGG ATGGAAAATG CCCAGTCTGT 3420
 45 GGAGGAAAGA TAGTGCTGAC AGTTAGCAAA GGAGCCATTG AAAAGTACTT GGGGACTGCC 3480
 AAGATGCTCG TAGCTAACTA CAACGTAAAG CCATATACAA GGCAGAGAAT ATGCTTGACG 3540

GAGAAGGATA TTGATTCACT CTTTGAGTAC TTATTCCCAG AAGCCCAGTT AACGCTCATT 3600
 GTAGATCCAA ACGACATCTG TATGAAAATG ATCAAGGAAA GAACGGGGGA AACAGTTCAA 3660
 5 GGAGGCCTGC TTGAGAACTT TAATTCCTCT GGAAATAATG GGAAGAAAAT AGAGAAGAAG 3720
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10 SEQ ID NO:5

SEQUENCE LENGTH: 8450

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

15 TOPOLOGY: linear

MOLECULAR TYPE: Genomic DNA

SEQUENCE DESCRIPTION:

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 CAATGAACGA AGGTGAACAT CAAATAAAGC TTGACGAGCT ATTGCGAAAAG TTGCTCCGAG 180
 CTAGGAAGAT ATTCAAAAAC AAAGATGTCC TTAGGCATAG CTATACTCCC AAGGATCTAC 240
 25 CTCACAGACA TGAGCAAATA GAAACTCTCG CCCAAATTTT AGTACCAGTT CTCAGAGGAG 300
 AAATCCATC AACATATTC GTTTATGGGA AGACTGGAAC TGGAAAGACT GTAAGTGTAA 360
 AATTTGTAAC TGAAGAGCTG AAAAGAATAT CTGAAAAATA CAACATTCCA GTTGATGTGA 420
 TCTACATTAA TTGTGAGATT GTCGATACTC ACTATAGAGT TCTTGCTAAC ATAGTTAACT 480
 30 ACTTCAAAGA TGAGACTGGG ATTGAAGTTC CAATGGTAGG TTGGCCTACC GATGAAGTTT 540
 ACGCAAAGCT TAAGCAGGTT ATAGATATGA AGGAGAGGTT TGTGATAATT GTGTTGGATG 600
 AAATTGACAA GTGGTAAAGA AGAGTGGTGA TGAGGTTCTC TATTCATTAA CAAGAATAAA 660
 TACTGAACCT AAAAGGGCTA AAGTGAGTGT AATTGGTATA TCAAACGACC TTAAATTTAA 720
 35 AGAGTATCTA GATCCAAGAG TTCTCTCAAG TTTGAGTGAG GAAGAGGTGG TATTCACCAC 780
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 40 AGATGCAAGA AAGGCACTTG ACCTTCTAAG AGTTGCAGGG GAAATAGCGG AAAGAGAAGG 960
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 45 GGAATTGTGC GAGTACATTG ACTTGGAACC TCTACCCAA AGAAGGATAA GTGATCTAAT 1200
 TAATGAGCTT GACATGCTTG GAATAATAAA TGCAAAAGTT GTTAGTAAGG GGAGATATGG 1260

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GAGGACAAAG GAAATAAGGC TTAACGTTAC CTCATATAAG ATAAGAAATG TGCTGAGATA 1320
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5 CTAATGGATG AATTTGTAAA ATCACTTCTA AAAGCTAACT ATCTAATAAC TCCCTCTGCC 1440
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10 GAGTCCCAAA AAGAGCAGTC TTATGAAGAG TCTTTTGGGA CTAAAGAAGA AATTTCCCAG 1680
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25 AGGGAGGCAT TTAAGGTTCT TCCAGATGCC GTCGTCGCTT TTAAGGGGGT GTATTCAAAG 2340
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 5 AGCTTTTAAA AGAGTATGAT AAGGAAATTG TTGCTTTAAA GATAGTTGAT GAGATAATTG 3480
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 10 CAATTAGGAG TTCTGGTGGA ACTGCTCAAG CTCTCAGTGT ACTTGTGGT GATTACGTTA 3720
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 25 ATCCAGCAAC AATGGTTTTG GTTGACGAGT TCTTGGCCAT TGGAACCTAA ATGAAAACCG 4380
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 30 AGAACAATCA AACTCTCCTT CCTGCAAACT ATGTAGAGGA GTGGTGGATC CAAGAGTTG 4620
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AGTGTGGCCA TGTAGGGCCT GAAACTCTCT GTCCCGAGTG TGGGATTAGG AAAGAGTTGA 5400
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 5 ATTCATGTCC AAAGTGCAAT GTGAAGCTAA AGCCATTAC AAAGAGGAAG ATAAAGCCCT 5520
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 30 CAAAGTACAG AAGGCCGCCC TTGGATGGAA AATGCCAGT CTGTGGAGGA AAGATAGTGC 6660
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 40 AACTTTTAAG TTCTTTCTTG AGAATAAATT CCCAGGTGGC TTAGAGAATG AAGATTGTGT 7080
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 45 GGCCGAAAAC AGTTTATCTC ATGGCAATAA GTGATGGGCT AACAAAGGTC AGAGAGATAG 7320
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CAACAAGCCA GTATCCCCTG GGATATCTAA TTGAAGGAAG CAAAAGAGTG GCTCACTTGG 7440
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TACGGGCCAT TTGCCACCGG GAAGACAAC TTTGCAATGC AGGTTGGATT ATTGAATGAG 7860
GGAAAAGTGG CTTATGTTGA TACTGAGGGG GGATTCTCCC CCGAAAGGTT AGCTCAAATG 7920
GCAGAATCAA GGAACCTGGA TGTGGAGAAA GCACTTGAAA AGTTCGTGAT ATTCGAACCT 7980
15 ATGGATTTAA ACGAGCAAAG ACAGGTAATT GCGAGGTTGA AAAATATCGT GAATGAAAAG 8040
TTTTCTTTAG TTGTGGTCGA CTCCTTTACG GCCCATTATA GAGCGGAGGG GAGTAGAGAG 8100
TATGGAGAAC TTTCCAAGCA ACTCCAAGTT CTTCACTGGA TTGCCAGAAG AAAAAACGTT 8160
20 GCCGTTATAG TTGTCAATCA AGTTTATTAC GATTCAAAC CAGGAATTCT TAAACCAATA 8220
GCTGAGCACA CCCTGGGGTA CAAAACAAAG GACATCCTCC GCTTTGAAAG GCTTAGGGTT 8280
GGAGTGAGAA TTGCAGTTCT GGAAAGGCAT AGGTTTAGGC CAGAGGGTGG GATGGTATAC 8340
TTCAAAATAA CAGATAAAGG ATTGGAGGAT GTAAAAACG AAGATTAGAG CCTGTCGTAG 8400
25 ACCTCCTGGG CAATCCTCAG CGTTGCCTTA TAGAGCTTCT CACTAATAAT 8450

SEQ ID NO:6

30 SEQUENCE LENGTH: 45

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

35 MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

CCGGAACCGC CTCCCTCAGA GCCGCCACCC TCAGAACCGC CACCC

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40 SEQ ID NO:7

SEQUENCE LENGTH: 17

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

45 TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic RNA)

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SEQUENCE DESCRIPTION:

GUUUUCCCAG UCACGAC

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SEQ ID NO:8

SEQUENCE LENGTH: 23

SEQUENCE TYPE: nucleic acid

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STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

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GATGAGTTCG TGTCCGTACA ACT

23

SEQ ID NO:9

SEQUENCE LENGTH: 22

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SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

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SEQUENCE DESCRIPTION:

ACAAAGCCAG CCGGAATATC TG

22

SEQ ID NO:10

SEQUENCE LENGTH: 22

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

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TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

TACAATACGA TGCCCCGTTA AG

22

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SEQ ID NO:11

SEQUENCE LENGTH: 32

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

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TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

5 SEQUENCE DESCRIPTION:

CAGAGGAGGT TGATCCCATG GATGAATTG TA

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SEQ ID NO:12

10 SEQUENCE LENGTH: 32

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

15 MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

TTTAGTGGGT GGTGCCCCATG GAGCTTCCAA AG

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25 Claims

1. A DNA polymerase characterized in that said DNA polymerase possesses the following properties:

- 30 1) exhibiting higher polymerase activity when assayed by using as a substrate a complex resulting from primer annealing to a single stranded template DNA, as compared to the case where an activated DNA is used as a substrate;
- 2) possessing a 3'→5' exonuclease activity;
- 3) being capable of amplifying a DNA fragment of about 20 kbp, in the case where polymerase chain reaction (PCR) is carried out using λ-DNA as a template under the following conditions:
- 35 PCR conditions:

- (a) a composition of reaction mixture: containing 10 mM Tris-HCl (pH 9.2), 3.5 mM MgCl₂, 75 mM KCl, 400 μM each of dATP, dCTP, dGTP and dTTP, 0.01% bovine serum albumin, 0.1% Triton X-100, 5.0 ng/50 μl λ-DNA, 10 pmole/50 μl primer λ1 (SEQ ID NO:8 in Sequence Listing), primer λ11 (SEQ ID NO:9 in Sequence Listing), and 3.7 units/50 μl DNA polymerase;
- 40 (b) reaction conditions: carrying out a 30-cycle PCR, wherein one cycle is defined as at 98°C for 10 seconds and at 68°C for 10 minutes.

- 45 2. The DNA polymerase according to claim 1, characterized in that said DNA polymerase exhibits a lower error rate in DNA synthesis as compared to Taq DNA polymerase.
3. The DNA polymerase according to claim 1 or 2, wherein the molecular weight as determined by gel filtration method is about 220 kDa or about 385 kDa.
- 50 4. The DNA polymerase according to any one of claims 1 to 3, characterized in that said DNA polymerase exhibits an activity under coexistence of two kinds of DNA polymerase-constituting protein, a first DNA polymerase-constituting protein and a second DNA polymerase-constituting protein.
- 55 5. The DNA polymerase according to claim 4, characterized in that the molecular weights of said first DNA polymerase-constituting protein and said second DNA polymerase-constituting protein are about 90,000 Da and about 140,000 Da as determined by SDS-PAGE, respectively.
6. The DNA polymerase according to claim 4 or 5, characterized in that said first DNA polymerase-constituting protein

which constitutes the DNA polymerase according to claim 4 or 5 comprises the amino acid sequence as shown by SEQ ID NO:1 in Sequence Listing, or is a functional equivalent thereof possessing substantially the same activity which results from deletion, insertion, addition or substitution of one or more amino acids in said amino acid sequence.

7. The DNA polymerase according to claim 4 or 5, characterized in that said second DNA polymerase-constituting protein which constitutes the DNA polymerase according to claim 4 or 5 comprises the amino acid sequence as shown by SEQ ID NO:3 in Sequence Listing, or is a functional equivalent thereof possessing substantially the same activity which results from deletion, insertion, addition or substitution of one or more amino acids in said amino acid sequence.
8. The DNA polymerase according to claim 4 or 5, characterized in that said first DNA polymerase-constituting protein which constitutes the DNA polymerase according to claim 4 or 5 comprises the amino acid sequence as shown by SEQ ID NO:1 in Sequence Listing, or is a functional equivalent thereof possessing substantially the same activity which results from deletion, insertion, addition or substitution of one or more amino acids in said amino acid sequence, and that said second DNA polymerase-constituting protein which constitutes the DNA polymerase according to claim 4 or 5 comprises the amino acid sequence as shown by SEQ ID NO:3 in Sequence Listing, or is a functional equivalent thereof possessing substantially the same activity which results from deletion, insertion, addition or substitution of one or more amino acids in said amino acid sequence.
9. A first DNA polymerase-constituting protein which constitutes the DNA polymerase according to claim 4 or 5, wherein said first DNA polymerase-constituting protein comprises the amino acid sequence as shown by SEQ ID NO:1, or an amino acid sequence resulting from deletion, insertion, addition or substitution of one or more amino acids in said amino acid sequence as a functional equivalent thereof possessing substantially the same activity.
10. A second DNA polymerase-constituting protein which constitutes the DNA polymerase according to claim 4 or 5, wherein said second DNA polymerase-constituting protein comprises the amino acid sequence as shown by SEQ ID NO:3, or an amino acid sequence resulting from deletion, insertion, addition or substitution of one or more amino acids in said amino acid sequence as a functional equivalent thereof possessing substantially the same activity.
11. A DNA containing a base sequence encoding the first DNA polymerase-constituting protein according to claim 9, characterized in that said DNA comprises an entire sequence of a base sequence encoding the amino acid sequence as shown by SEQ ID NO:1 in Sequence Listing, or a partial sequence thereof, or that said DNA encodes a protein having an amino acid sequence resulting from deletion, insertion, addition or substitution of one or more amino acids in the amino acid sequence of SEQ ID NO:1 and possessing a function as the first DNA polymerase-constituting protein.
12. A DNA containing a base sequence encoding the first DNA polymerase-constituting protein according to claim 9, characterized in that said DNA comprises an entire sequence of the base sequence as shown by SEQ ID NO:2 in Sequence Listing or a partial sequence thereof, or that said DNA comprises a base sequence capable of hybridizing thereto under stringent conditions.
13. A DNA containing a base sequence encoding the second DNA polymerase-constituting protein according to claim 10, characterized in that said DNA comprises an entire sequence of a base sequence encoding the amino acid sequence as shown by SEQ ID NO:3, or a partial sequence thereof, or that said DNA encodes a protein having an amino acid sequence resulting from deletion, insertion, addition or substitution of one or more amino acids in the amino acid sequence of SEQ ID NO:3 and possessing a function as the second DNA polymerase-constituting protein.
14. A DNA containing a base sequence encoding the second DNA polymerase-constituting protein according to claim 10, characterized in that said DNA comprises an entire sequence of the base sequence as shown by SEQ ID NO:4 in Sequence Listing or a partial sequence thereof, or that said DNA comprises a base sequence capable of hybridizing thereto under stringent conditions.
15. A method for producing a DNA polymerase, characterized in that the method comprises culturing a transformant containing both gene encoding the first DNA polymerase-constituting protein according to claim 11 or 12, and gene encoding the second DNA polymerase-constituting protein according to claim 13 or 14, and collecting the DNA polymerase from the resulting culture.

16. A method for producing a DNA polymerase, characterized in that the method comprises culturing a transformant containing gene encoding the first DNA polymerase-constituting protein according to claim 11 or 12, and a transformant containing gene encoding the second DNA polymerase-constituting protein according to claim 13 or 14, separately; mixing DNA polymerase-constituting proteins contained in the resulting culture; and collecting the DNA polymerase.

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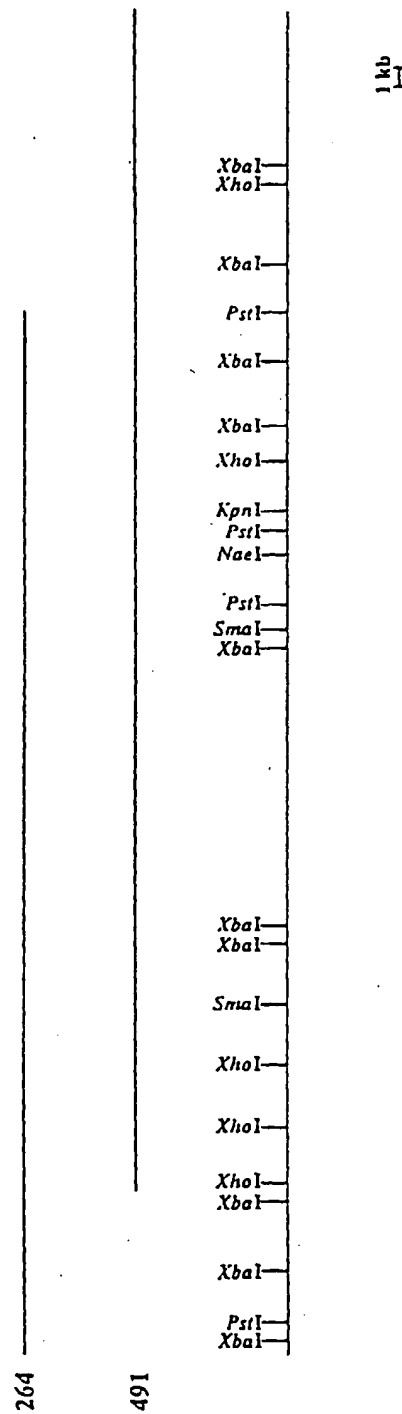


FIG. 1

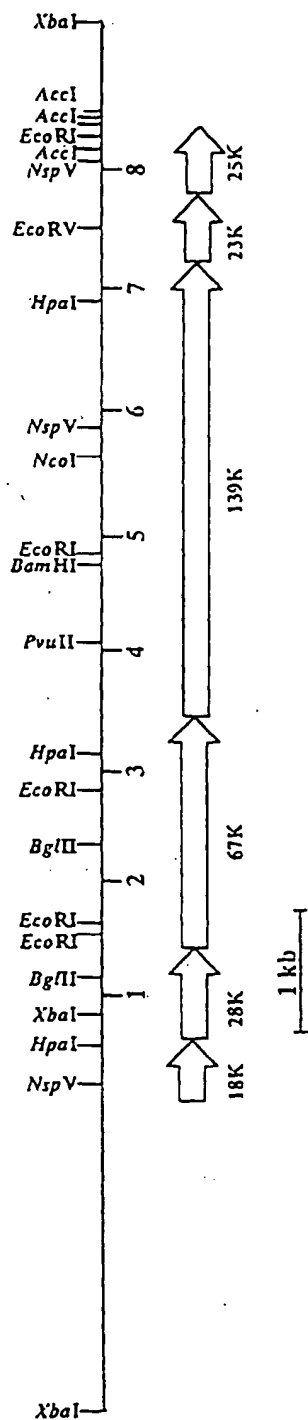


FIG. 2

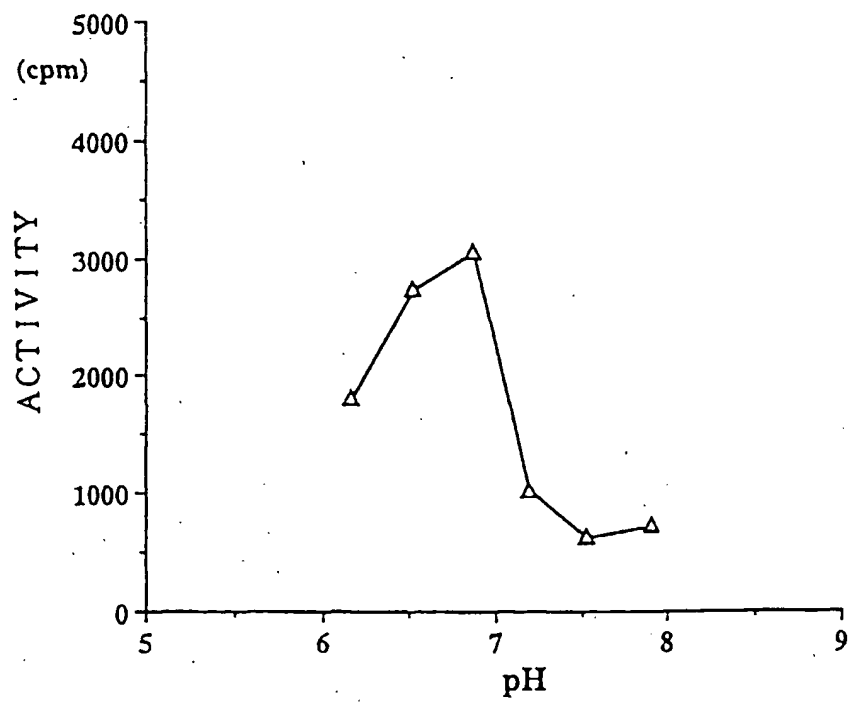
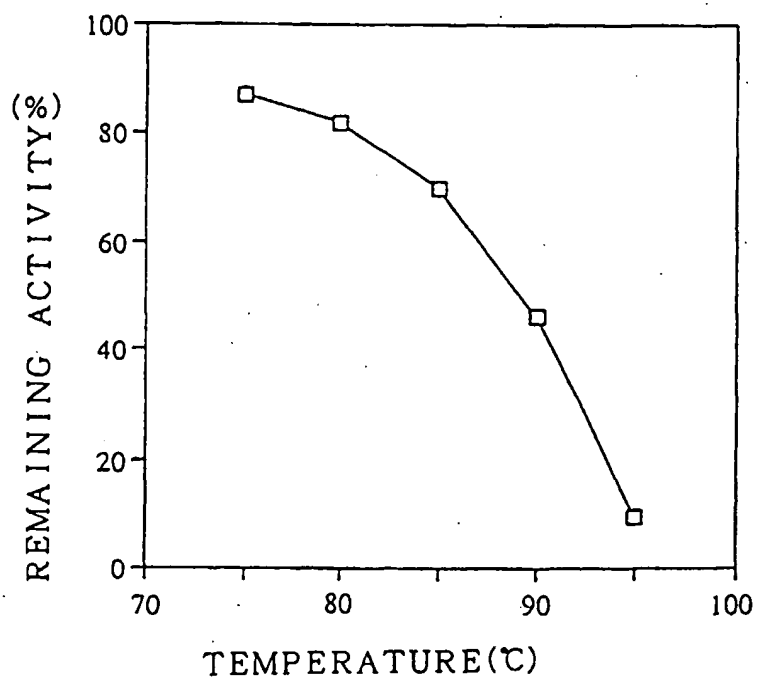


FIG. 3



F I G. 4

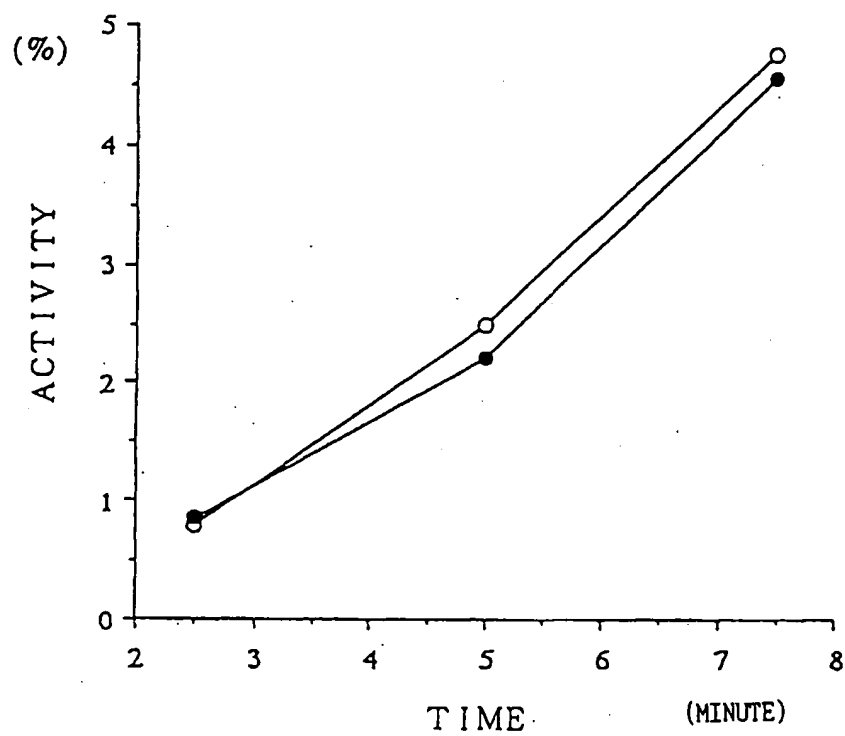


FIG. 5

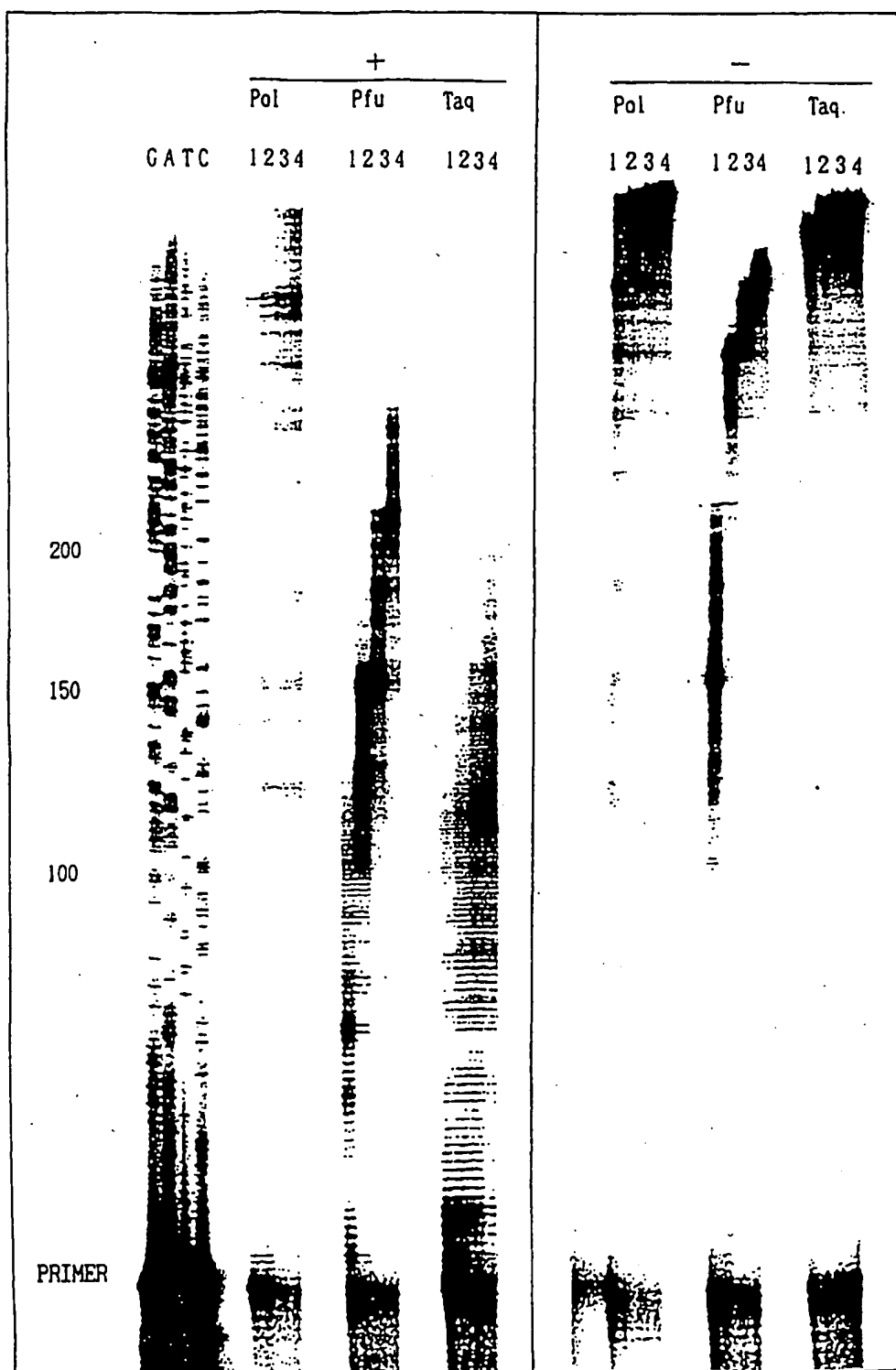


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP96/03869

A. CLASSIFICATION OF SUBJECT MATTER		
Int. C1 ⁶ C12N15/54, C12N9/12		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
Int. C1 ⁶ C12N15/54, C12N9/12		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
CAS ONLINE, BIOSIS, WPI/WPI,L		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Anal. Biochem. 231(1) 1995 Ju Jingyue et al. "Design and synthesis of fluorescence energy transfer dye-labeled primers and their application for DNA sequencing and analysis" p. 131-140	1 - 16
A	WO, 9426766, A (Barnes, Wayne M.), November 24, 1994 (24. 11. 94) & US, 543619, A & EP, 693078, A	1 - 16
	WO, 9317127, A (Oregon State Board of Higher Education), September 2, 1993 (02. 09. 93) & US, 5470724, A	1 - 16
A	J. Clin. Chem. Clin. Biochem. 28(1) 1990 Linz, Ute et al. "Systematic studies on paramaters influencing the performance of the polymerase chain reaction" p. 5-13	1 - 16
X/A	WO, 9209689, A (Stratagene),	1 - 16
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "A" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
April 1, 1997 (01. 04. 97)		April 15, 1997 (15. 04. 97)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP96/03869

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	June 11, 1992 (11. 06. 92) (Family: none)	
A	EP, 669401, A (Hoffman La Roche Inc.), August 30, 1995 (30. 08. 95) & JP, 8038198, A & US, 5512462, A	1 - 16
A	WO, 9516028, A (Stratagene), June 15, 1995 (15. 06. 95) & US, 5556772, A	1 - 16
X/AL	Nucleic Acids Res. 20(7) 1992 Forterre, Patrick "The DNA polymerase from the archaeobacterium pyrococcus furiosus does not testify for a specific relationship between archaeobacteria and eukaryotes" p. 1881	1/2-16
X/A	Gene 108(1) 1991 Lundberg, Kelly S. et al. "High-fidelity amplification using a thermostable DNA polymerase isolated from pyrococcus furiosus" p. 1-6	1-2/3-16
A	J. Bacteriol. 177(8) 1995 Uemori, Takashi et al. "The hyperthermophilic archaeon pyrodictium ocultium has two alpha-like DNA polymerase" p. 2164-2177	1 - 16

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